NITRIC OXIDE AND CARDIAC MYOCYTE CONTRACTION

by

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Dovehouse Street
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A thesis submitted to the University of Edinburgh
for the degree of Doctor of Medicine

1993

Dedicated
to the memory of my father,

Conall A. Brady

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Declaration of Originality

All the hypotheses addressed in the work of this thesis are my own. I conceived of and developed the methodology where new experimental methods were required, *i.e.* the coculture studies; experiments involving nitric oxide gas; and endotoxin studies. I modified existing methods for the other studies involving cardiac myocytes. Technical assistance was available for preparation of cells. I performed all of the experiments described in this thesis.

13th September 1993

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ABSTRACT

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Nitric oxide is involved in the function of many cell types, including nerve, muscle, leucocytes and endothelium. Whether nitric oxide is implicated in the contractile function of isolated cardiac ventricular myocytes forms the major part of the work of this thesis.

Contracting guinea-pig cardiac myocytes were studied in isolation *in vitro* using a videomicroscopy length detection system.

Studies are presented which establish that nitric oxide attenuates contractility of cardiac ventricular myocytes, both when it is derived from exogenous sources, and when nitric oxide is released from adjacent endothelium in coculture with cardiac myocytes. The coronary microcirculation is in close proximity to cardiac myocytes within the myocardium, thus endothelium-derived nitric oxide may have an important tonic effect on myocardial contractility. This may be particularly important when the diffusing distance from endothelial cell to myocyte is altered in disease states.

Myocardial contractility is impaired in endotoxic shock. The hypothesis that this is caused by production of nitric oxide within cardiac myocytes is examined. A model of endotoxic shock was developed. Contractility of cardiac myocytes was substantially impaired. Much of this impairment was caused by nitric oxide production within the cardiac myocytes themselves. Inhibition of nitric oxide synthesis in these cells restored contractility towards normal. Healthy myocytes did not produce effective amounts of nitric oxide. Induction of nitric oxide synthase activity within cardiac myocytes may account for much of the depressed contractility of endotoxic heart failure.

Myocardial contractility is impaired following ischaemia-reperfusion. Experiments examining myocyte behaviour in this situation are presented, but whether activation of nitric oxide synthase contributes to the impaired contractility of myocytes following ischaemia is not established. Studies to define the direct action of neutrophils on cardiac myocytes are presented, and reasons why these experiments did not generate definitive answers are discussed.

Nitric oxide is an important modulator of cardiac myocyte contraction. It may have a role in the regulation of myocardial contractility. Production of nitric oxide within myocytes may contribute to the depressed contractility of endotoxic heart failure.

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To Professor Philip Poole-Wilson, under whose supervision the work of this thesis was performed, and whose encouragement and enthusiasm supported me throughout my research, I shall always be indebted.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BK	bradykinin
$[Ca^{2+}]_o$	calcium concentration in extracellular/perfusing fluid
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CD	cluster of differentiation
CGRP	calcitonin gene related peptide
cNOS	constitutive nitric oxide synthase
D-arg	D-arginine
D-NAME	N^{ω}-nitro-D-arginine methyl ester
Dex	dexamethasone
EC	endothelial cell(s)
EDRF	endothelial-derived relaxing factor
ELAM-1	endothelial-leucocyte adhesion molecule-1
FACS	fluorescence activated cell sorting
FMLP	formyl-methionyl-leucyl-phenylalanine
GMP	guanosine monophosphate
GMP-140	granule membrane protein-140
GTN	glyceryl trinitrate
HEPES	N-[2-Hydroxyethyl]piperazine-n'-]2-ethanesulfonic acid, (sodium salt)
I_{Ca}	L-type inward calcium channel current
iC3b	inactivated complement fragment 3b
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
Indo	indomethacin
iNOS	inducible nitric oxide synthase

IP_3	inositol 1,4,5-trisphosphate
ISDN	isosorbide dinitrate
KH	Krebs-Henseleit solution
L-arg	L-arginine
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NMMA	N^{ω}-methyl-L-arginine
LPS	lipopolysaccharide (endotoxin)
LTB_4	leukotriene B_4
MB	methylene blue
$[Na^+]_i$	intracellular sodium concentration
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NANC	non-adrenergic, non-cholinergic
NO	nitric oxide
NO_2, NO_3	nitrite, nitrate
NOS	nitric oxide synthase
NS	not significant
NTA	nitrilotriacetic acid
PAF	platelet activating factor
PDGF	platelet derived growth factor
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PMA	phorbol myristate acetate
PMN	polymorphonuclear leucocyte (neutrophil)
PTCA	percutaneous transluminal coronary angioplasty
PTR	time from peak contraction to 90% relaxation
SEM	standard error of the mean
SNP	sodium nitroprusside
SR	sarcoplasmic reticulum
TGF-β	transforming growth factor-β
TNFα	tumour necrosis factor α
TTP	time to peak contraction

CHAPTER 1: INTRODUCTION

1.1 NITRIC OXIDE BIOLOGY

Nitric oxide is an inorganic radical gas. It is also one of the most important modulators of cellular function in biology. Only since the 1970s has this importance become apparent.

Its existence has long been known to chemists as a simple molecule: N=O, with an unpaired electron. In its pure form, in solid or liquid phase, nitric oxide exists as a dimer for chemical stability. In the environment, the odd electron makes it a highly reactive species. Nitric oxide reacts readily with oxygen to form nitrogen dioxide, a powerful oxidising agent. Nitric oxide and other reactive oxides of nitrogen are produced by plants, by combustion of hydrocarbons in motor vehicles and jet aircraft, and combustion of most other biological materials (Halliwell & Gutteridge, 1993). This introduction begins with a brief history of how the discovery of the importance of nitric oxide to biology evolved.

1.1.1. Discovery of the biological importance of nitric oxide

Four independent cornerstones of investigation led to the discovery of the importance of nitric oxide in biological systems (Nathan, 1992), illustrated in Figure 1.

(i) Mammalian cells generate a reactive nitrogen oxide from the guanidino end of the amino acid, L-arginine.

(ii) These reactive nitrogen oxides mediate a physiological reaction in macrophages. Chemically modified L-arginine analogues block the synthesis of nitrogen oxides.

(iii) Another tissue, the endothelium, also produces the reactive nitrogen oxide, nitric oxide. Its action within the vasculature had already been the subject of intensive research by vascular biologists, who had identified some of its functions but not its nature.

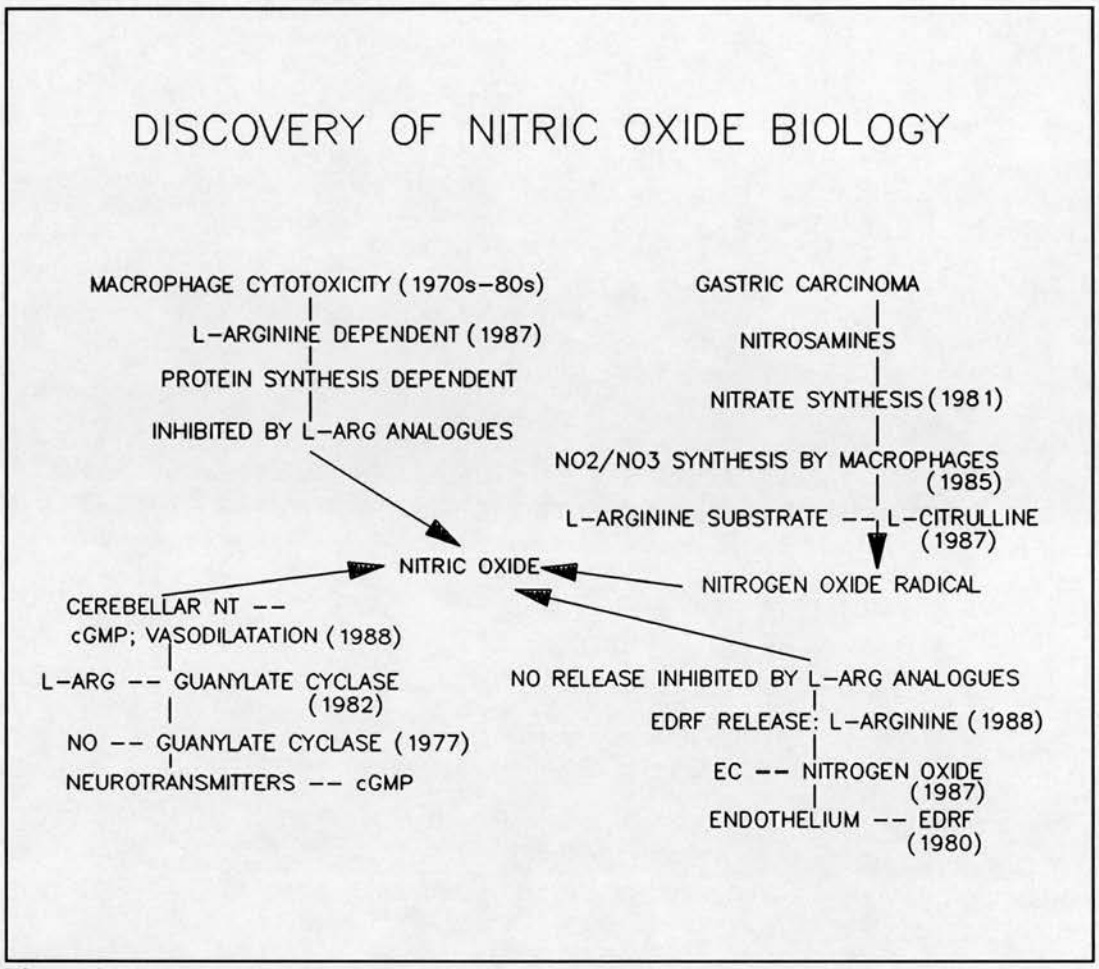


Figure 1

(iv) Nitric oxide is produced by neural tissue and has properties of a neurotransmitter.

Macrophages as a source of nitric oxide.

Interest in biological nitrates developed following the association of nitrosamines with carcinoma of the stomach. Attempts to study this in animal models and human subjects showed that mammals synthesise nitrates (Green *et al.*, 1981b; Green *et al.*, 1981a). At first it was considered that intestinal bacteria were the sole source of these nitrates. But studies with germ-free rats showed that these animals also produced nitrogen oxides. An important observation was made when a human subject became ill with intercurrent infection during one study (Green *et al.*, 1981a). Excretion of NO_3- became markedly elevated and this finding was confirmed in

animal experiments (Wagner *et al.*, 1983; Stuehr & Marletta, 1985). It appeared that macrophages accounted at least in part for the excess production of nitrogen oxides. Explanted macrophages were able to secrete these substances (Stuehr & Marletta, 1985), generated from oxidation of a guanidino nitrogen from the amino acid, L-arginine (Iyngar *et al.*, 1987). This oxidation yielded the amino acid citrulline, and a reactive compound, more reactive than either nitrite or nitrate, which could nitrosate amines to toxic nitrosamines (Miwa *et al.*, 1987). Thus the observation was made that mammalian cells could generate reactive nitrogen oxides from L-arginine.

Independently, other researchers were studying the oxidative injury macrophages cause to tumour cells and fungi. Hibbs et al had been studying this field since the early 1970s (Hibbs *et al.*, 1971; Hibbs *et al.*, 1972; Hibbs *et al.*, 1990b) and showed in 1987 that macrophage cytotoxicity required L-arginine, and that modification of the L-arginine molecule blocked both nitrite production and cytotoxicity of macrophages (Hibbs *et al.*, 1987). The production of nitrogen compounds also required protein synthesis, implying induction of an enzyme within the macrophages(Stuehr & Marletta, 1987). The development of analogues of L-arginine which inhibit synthesis of nitrogen oxides paved the way for an explosion in nitric oxide research.

Endothelium as a source of nitric oxide.

Furchgott and Zawadski had shown in 1980 (Furchgott & Zawadzki, 1980) that endothelium was not merely an inert lining of a hollow tube, but that it influenced its adjacent smooth muscle in the vascular wall. The identification of their endothelial-derived relaxing factor (EDRF) as nitric oxide evolved over the following eight years, although as early as 1977 Arnold et al had shown nitric oxide capable of activating guanylate cyclase in different tissues, the enzyme now known to be the target for nitric oxide in smooth muscle (Arnold *et al.*, 1977). In mid-1986 Furchgott and Ignarro independently suggested that EDRF might be nitric oxide (Ignarro *et al.*, 1988). In 1987, two groups showed that stimulated endothelium released a nitrogen oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987) which was neither nitrite or nitrate, although a chemiluminescent assay failed to identify which nitrogen oxide was active (Palmer *et al.*, 1987). In 1988, Palmer et al and Schmidt et al then showed that

EDRF release from cultured cells required L-arginine (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988), and that same year Palmer et al demonstrated that release of nitric oxide from endothelium was inhibited by analogues of L-arginine (Palmer *et al.*, 1988b).

Neural tissue as a source of nitric oxide.

Neurobiologists had known since the early 1970s that some neurotransmitters elevated cyclic GMP in neural tissue (Ferrendelli *et al.*, 1974). In 1977 Miki et al showed that nitric oxide stimulated the guanylate cyclase enzyme in mouse cerebral cortex (Miki *et al.*, 1977). In 1982 L-arginine was shown to activate guanylate cyclase in neuroblastoma cells (Deguchi & Yoshioka, 1982) and in 1988 cerebellar tissue was shown to release a mediator with properties like nitric oxide, which both elevated cyclic GMP and relaxed vascular smooth muscle (Garthwaite *et al.*, 1988). Release of nitric oxide and citrulline by neural tissue was inhibited by an analogue of L-arginine, evidence that synaptic cells contained the nitric oxide synthase enzyme (Knowles *et al.*, 1989). Thus nervous tissue, as well as leucocytes and endothelium, was shown to employ nitric oxide as a chemical mediator.

Moncada demonstrated recently the importance in terms of phylogenetics and evolution of nitric oxide biology (invited lecture to the American Heart Association, November 1991, Anaheim, USA). Not only do all mammals examined employ nitric oxide as an intercellular mediator, but even primitive invertebrates use nitric oxide in their circulatory "blood-like" cells as a chemical messenger, and probably as a cytotoxic agent too.

1.1.2. **Biosynthesis of nitric oxide.**

Nitric oxide is synthesised from the amino acid, L-arginine. It was believed that cells from different tissues had different synthetic pathways, but recent work has resolved this. Figure 2 shows the pathway of nitric oxide production from L-arginine (modified from Nathan)(Nathan, 1992). Both oxidation steps are catalysed by the enzyme, nitric oxide synthase. NADPH donates three electrons and molecular oxygen provides two more.

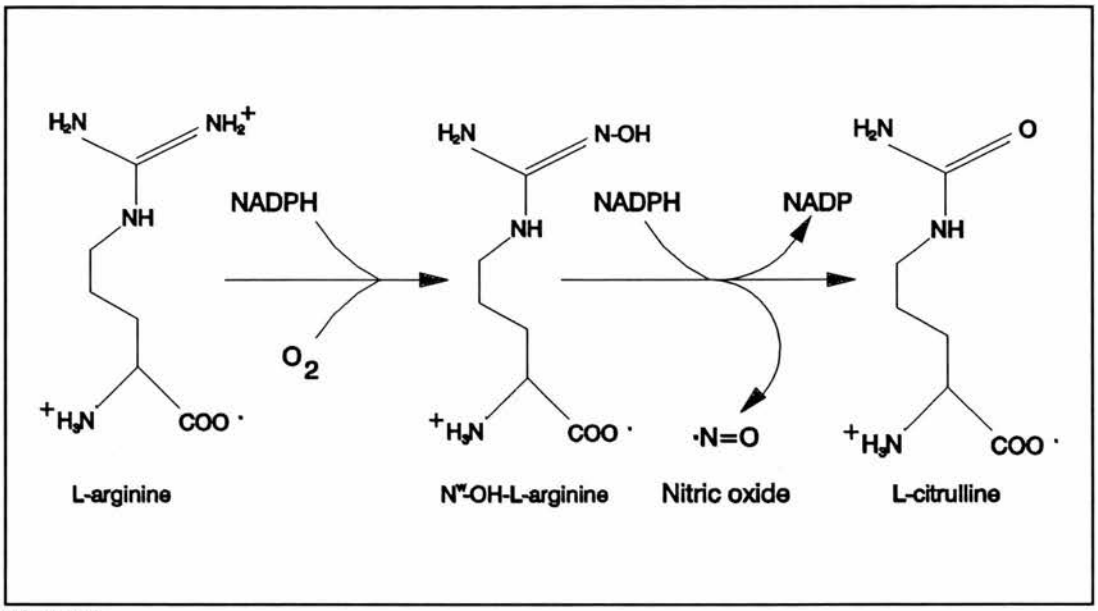


Figure 2

Different nitric oxide synthase enzymes have been isolated from diverse tissues and species. At present, no nomenclature classifies them satisfactorily. Three enzyme types for which cDNAs have been reported at this time are listed below (Nathan, 1992), shown in Table 1.

	constitutive NOS (cNOS)	constitutive NOS (cNOS)	inducible NOS (iNOS)
Source	rat cerebellum	bovine aortic endothelium	mouse macrophage
Mechanism of activation	elevated $[Ca^{2+}]_i$ → binding of calmodulin	elevated $[Ca^{2+}]_i$ → binding of calmodulin	induction of new mRNA

Table 1

Activation of the constitutive nitric oxide synthase (cNOS) enzymes follows elevation of $[Ca^{2+}]_i$, which causes the calcium binding protein, calmodulin, to adhere to and activate constitutive nitric oxide synthase (Busse & Mulsch, 1990; Nathan, 1992). These nitric oxide synthases are thus susceptible to agonists that elevate Ca^{2+}, and are termed Ca^{2+}-dependent by some authors (Moncada *et al.*, 1991).

Activation of mouse macrophage nitric oxide synthase is not controlled by changes in $[Ca^{2+}]_i$, since this type of nitric oxide synthase binds calmodulin tightly at resting intracellular levels of calcium (Cho *et al.*, 1992). Induction of new protein

is required to synthesise this nitric oxide synthase, hence it is termed inducible (Xie *et al.*, 1992) whereas nitric oxide synthase in brain and endothelium exists in resting cells and is termed constitutive (Moncada & Higgs, 1991). Human endothelial constitutive nitric oxide synthase has recently been cloned by T Michels in Boston (Nathan, 1992). It has 94% homology with the bovine constitutive nitric oxide synthase amino acid sequence.

1.1.3. **Distribution and activation of nitric oxide synthases**

At the time of writing nitric oxide synthases have been divided broadly into constitutive enzymes and inducible ones. As Table 2 below shows, constitutive nitric oxide synthases are activated rapidly by a variety of inflammatory or pharmacological stimuli. Inducible nitric oxide synthase enzymes require induction of mRNA and protein synthesis and this process is stimulated by inflammatory cytokines.

	cNOS enzymes	iNOS enzymes
sources	Endothelial cells Some central neurons Some peripheral NANC neurons Astrocytes Circulating neutrophils Mast cells Platelets Pancreatic ß islet cells Cardiac myocytes	Macrophages Kupffer cells Hepatocytes Vascular smooth muscle cells Fibroblasts Endothelial cells Activated neutrophils Articular chondrocytes Cardiac myocytes*
rapid activators	acetylcholine, bradykinin, ADP, thrombin, endotoxin, FMLP, leukotrienes, PAF, some amino acids, calcium ionophores, PMA, electrical stimulation	none known
inducers of mRNA for	none known	Endotoxin (lipopolysaccharide) Interferon Combinations of TNF, interferons, IL-1 and LPS

Table 2

** The demonstration and function of nitric oxide synthase activity within cardiac myocytes is the central finding of this thesis*

Agents which stimulate nitric oxide production within seconds or minutes of exposure act on existing constitutive nitric oxide synthase. Many of the rapid activators listed elevate $[Ca^{2+}]_i$, causing calmodulin to bind to nitric oxide synthase. Tissues which contain inducible nitric oxide synthase take several hours to generate nitric oxide. This process is blocked by inhibitors of DNA transcription or mRNA translation, thus new protein synthesis occurs. Once induced, inducible nitric oxide synthase generates nitric oxide for many hours or even days (Xie *et al.*, 1992). How this production is turned off is not known, but mechanisms to regulate nitric oxide production once initiated likely exist, but have yet to be discovered.

A range of substances have been developed or discovered to inhibit nitric oxide synthase. These include:-

(i) Substrate inhibitors (Moncada *et al.*, 1991): (analogues of L-arginine)- N^{ω}-methyl-

L-arginine (L-NMMA); N^{ω}-nitro-L-arginine; N^{ω}-nitro-L-arginine methyl ester (L-NAME); N^{ω}-amino-L-arginine.

(ii) Flavoprotein binders (Stuehr *et al.*, 1991). Diphenylene iodinium binds to flavoprotein cofactors involved in nitric oxide synthase function. This agent also binds to other cellular flavoproteins and is thus not a useful therapeutic agent.

(iii) Calmodulin binders (Bredt & Snyder, 1990). These pharmacological tools are available but since calmodulin is involved in many cellular processes, they are not useful therapeutically.

(iv) Carbon monoxide (Nathan, 1992). Nitric oxide synthase contains a haem moiety, to which carbon monoxide can bind and render inactive. Clearly this is not useful as therapy. Intriguingly, emerging evidence points to physiological roles for carbon monoxide not dissimilar to those of nitric oxide. Both substances are simple gases, both act like neurotransmitters, both bind to haemoglobin and both activate guanylate cyclase (Verma *et al.*, 1993). Much will be heard about carbon monoxide in the near future.

Apart from the calmodulin binders the above inhibitors affect both constitutive nitric oxide synthase and inducible nitric oxide synthase. Induction of inducible nitric oxide synthase can be effectively prevented by corticosteroids (Di Rosa *et al.*, 1990), and by certain cytokines: transforming growth factor -β (TGF-ß), and the interleukins IL-4 and IL-10 (Ding *et al.*, 1990).

1.1.4. **Intracellular site of action of nitric oxide in the vasculature**

In vascular tissue the principal target for nitric oxide is the soluble guanylate cyclase enzyme (Ignarro, 1990). Guanylate cyclase is activated by nitrosation of its haem moiety by nitric oxide. Elevation of intracellular cyclic GMP (cGMP) concentrations brings about vasorelaxation by mechanisms which are partially characterised, but which must ultimately involve cellular handling of cytosolic calcium.

Free cytosolic Ca^{2+} causes smooth muscle contraction by binding to calmodulin, and this complex binds to and activates myosin light chain kinase which phosphorylates the myosin light chain, permitting activation of Mg^{2+}-dependent

ATPase on the myosin cross bridge of actin. Hydrolysis of ATP follows and tension is developed by conformational changes of cross bridges and relative movements of actin and myosin. Tension is regulated by the number and rate of turnover of active cross bridges (Stull & Sanford, 1981). Thus, decreased levels of free cytosolic Ca^{2+} result in less contraction.

CGMP probably enhances phosphorylation of key proteins involved in handling Ca^{2+}, perhaps along with direct inhibition of Ca^{2+} binding to contractile proteins. CGMP and its dependent protein kinase inhibit:

(i) Ca^{2+} entry into cells through receptor-operated channels (Godfraind, 1980; Malta *et al.*, 1986);

(ii) Ca^{2+} release from sarcoplasmic reticulum (Kobayashi *et al.*, 1985);

(iii) production of inositol triphosphate (IP_3) (Rapoport, 1986);

(iv) activation of sarcolemmal Ca^{2+}-ATPase and therefore increase Ca^{2+} extrusion from cells (Popescu *et al.*, 1985).

1.1.5. **Intracellular targets of nitric oxide in other cell types**

Macrophages exert their cytotoxic action on tumour cells by release of nitric oxide which inhibits Fe-S proteins such as NADH (Nathan, 1992). Nitric oxide also inhibits tumour cell DNA synthesis (Hibbs *et al.*, 1990a). Nitric oxide also reacts with superoxide anion in a reaction that can either detoxify both molecules, or generate the powerful oxidants, nitrogen dioxide and hydroxyl radical (Beckman *et al.*, 1990).

1.2 **ACTIONS OF NITRIC OXIDE IN THE VASCULATURE**

1.2.1. **Role of endothelium in the control of vascular tone**

The human body contains a great deal of endothelium - about 1000-1500 grams. The lungs alone account for about a third of this tissue (Warren, 1990b). The surface area of the body's endothelium would cover an area equal to the size of

six tennis courts (Henderson, 1991). Yet only since the mid 1960s has endothelium been considered as little more than an inert lining of blood vessels, and only over the last 13 years has its fundamental importance in the control of vascular homeostasis been recognised.

That blood flow could be regulated by generation of locally acting autacoids within the vasculature became apparent during the 1960's and 70's. Renin-like activity was detected in vascular walls (Gould *et al.*, 1964; Ryan *et al.*, 1976), and the pharmacology of prostaglandins and other autacoids developed (Sparks & Belloni, 1978).

In 1980 in their now classic paper Furchgott and Zawadski demonstrated the obligatory role of the endothelium in the relaxation of vascular smooth muscle by acetylcholine. This finding explained the paradox of acetylcholine behaviour in experimental preparations: that it had disparate effects, either vasodilatation or vasoconstriction *in vitro*, yet was a vasodilator *in vivo*. While acetylcholine relaxed precontracted rabbit aortic rings it had no effect on, or even increased contraction of, precontracted aortic strips. Furchgott and Zawadski noticed that endothelial damage was occurring in the preparation of aortic strips, and they postulated that acetylcholine was acting on an endothelial receptor to release substance(s) which mediated its vasodilator effect. The humoral nature of this relaxing substance, termed endothelium-derived relaxing factor (EDRF), was proven in an experiment where deendothelialised aorta was sandwiched, intima to intima, against aorta with endothelium intact (Furchgott, 1988).

Many investigators have confirmed and studied this phenomenon further. A wide range of physiological, chemical and physical agents act in whole or in part by releasing EDRF from endothelium from large arteries, microvessels and veins. These are summarised in Table 3.

STIMULUS	SPECIES	VESSEL	REFERENCE
Acetylcholine	rabbit rabbit	aorta coronary	(Furchgott & Zawadzki, 1980) (Griffith *et al.*, 1984)
Noradrenaline	dog	coronary	(Cocks & Angus, 1983)
ATP, ADP	rabbit dog	aorta femoral	(Furchgott, 1988) (De Mey & Vanhoutte, 1981)
Serotonin	dog	coronary	(Cohen *et al.*, 1984)
Thrombin	dog		(DeMey & Vanhoutte, 1982)
Substance P	rabbit dog	aorta coronary	(Zawadzki *et al.*, 1981) (Cocks & Angus, 1983)
Bradykinin	dog bovine	pulmonary artery	(Chand & Altura, 1981) (Ignarro *et al.*, 1988)
Adenosine	pig	aorta	(Gordon & Martin, 1983)
Vasoactive intestinal polypeptide	rat	aorta	(Davies & Williams, 1983)
Histamine	rat	aorta	(Van de Voorde & Leusen, 1983)
Vasopressin	dog	coronary	(Katusic *et al.*, 1984)
Calcitonin gene-related peptide	rat	aorta	(Brain *et al.*, 1985)
A23187	rabbit human	aorta coronary	(Zawadzki *et al.*, 1981) (Ginsburg & Zera, 1980)
Vascular smooth muscle factor	bovine	aorta	(Warren *et al.*, 1990)
Flow (shear stress)	dog dog dog rabbit	coronary femoral coronary ear	(Holtz *et al.*, 1983) (Pohl *et al.*, 1986) (Inque *et al.*, 1989) (Griffith *et al.*, 1984)
Oxygen (hypoxia)	bovine dog	coronary coronary	(Roberts *et al.*, 1981) (Busse *et al.*, 1983)

Table 3

These agents can be grouped into neurotransmitters, platelet products and coagulation factors, local hormones and physicochemical stimuli. Thus intravascular agents act by diffusion across the cell membrane, while agents released from autonomic nerve endings on smaller arteries may exert their action after diffusion from nerve terminal to endothelial cell (Henderson, 1991).

Many of the agents listed have vasoconstrictor properties in the absence of endothelium. This is particularly important when endothelium is damaged or removed, for example during percutaneous transluminal coronary angioplasty (PTCA) (Brady & Warren, 1991).

There is evidence for the existence of at least one other endothelium derived relaxing factor in addition to nitric oxide and prostacyclin. Taylor and Weston showed that stimulation of endothelium by acetylcholine caused membrane hyperpolarisation and this was not inhibited by haemoglobin or methylene blue (Taylor & Weston, 1988). The physiology of this unidentified factor, termed endothelium derived hyperpolarising factor, has not been defined.

Not only does endothelium produce factors which affect contraction of smooth muscle, but vascular smooth muscle itself releases an as yet unidentified factor which causes sustained release of nitric oxide from endothelium (Warren *et al.*, 1990). This substance was not characterised in this study with which I was involved, and its role in vascular physiology is not yet established, although this is being addressed by current work.

1.2.2. **Nitric oxide and blood flow**

The most important stimulus to EDRF release may well shear stress on the endothelial luminal surface, secondary to flow of blood. Shear stress exerted on endothelium varies considerably throughout the vasculature, since the characteristics of blood flow depend in part on vessel size, shape and deformability. Flow induced dilatation, long known but unexplained by physiologists, is substantially accounted for by mechanisms involving release of nitric oxide from endothelium. Rodbard in 1975 suggested that endothelium might sense alterations in flow as changes in shear stress

(Rodbard, 1975). This was finally shown by experiments where removal of endothelium markedly attenuated responsiveness of coronary arteries to changes in flow rate (Holtz *et al.*, 1983; Pohl *et al.*, 1986), and reactive hyperaemia was also inhibited (Inque *et al.*, 1989). This is supported by the finding that the non-specific inhibitor of guanylate cyclase, methylene blue, or haemoglobin, which binds nitric oxide, block flow dependent dilatation in dog femoral (Pohl *et al.*, 1986) and rabbit ear respectively (Griffith *et al.*, 1984). Griffith et al also found that flow dependent release of EDRF was greatest in vessels of approximately 150 μm diameter, where the resistance and shear force is maximal. Pulsatile flow also enhances release of EDRF, as well as of PGI_2 (Pohl *et al.*, 1985; Rubanyi *et al.*, 1986). Endothelium may sense shear stress by a K^+ channel sensitive to flow (Olesen *et al.*, 1988). Whether this causes release of nitric oxide is not yet known.

1.2.3. **Nitric oxide and hypoxia**

Much of the vasodilatation of systemic blood vessels supplying ischaemic tissue is caused by accumulation of metabolites. But recent studies have shown that vasoactive substances from endothelium probably contribute to vascular changes brought about by hypoxia. Nitric oxide, PGI_2 and PGE_2 are all released from hypoxic cultured endothelial cells (Roberts *et al.*, 1981) (Peach *et al.*, 1985) suggesting that oxygen sensors are located on endothelium (Bassenge & Busse, 1988). Park et al have recently shown that nitric oxide, adenosine and vasodilatory prostaglandins are all involved in hypoxic coronary vasodilatation (Park *et al.*, 1992).

1.2.4. **Actions of nitric oxide in vessels of different calibre**

The presence of nitric oxide activity has been demonstrated in every blood vessel studied, from large arteries to the microvasculature and the venous system (Henderson, 1991). But different vascular beds show marked differences in EDRF activity, both in response to nitric oxide and to its release (Christie & Lewis, 1988). Since many stimulants of EDRF release also act directly on smooth muscle, the effect on vascular tone depends on the relative strengths of the EDRF mediated dilatation

and the direct effect on vascular smooth muscle (Henderson, 1991).

EDRF activity differs between arteries and veins (DeMey & Vanhoutte, 1982). Veins release less EDRF for a particular stimulus. There are many differences between the two tissues, not only in their structure, but probably in their receptors for stimulants to EDRF release. That organic nitrovasodilators have a more potent effect on veins than arteries likely reflects greater activity of the enzymes necessary to generate nitric oxide from these drugs (Kawamoto *et al.*, 1990).

1.2.5. **Release of nitric oxide from microvessels**

Microvessels from different regions of the body tonically release nitric oxide. NO has been shown to be a vasodilator in several different microvascular preparations (Warren *et al.*, 1992; Gardiner *et al.*, 1990; Ekelund & Mellander, 1990; Persson *et al.*, 1990). Infusion of inhibitory analogues of L-arginine elevate blood pressure substantially, by inhibiting basal release of EDRF from resistance vessels (Rees *et al.*, 1989). In the human forearm a similar study caused reduction in arterial flow for the same reason (Vallance *et al.*, 1989). As mentioned in 1.2.2., there is an important relationship between flow induced shear stress and EDRF release from resistance vessels.

The heart is rich in microvascular endothelium (see section 1.4). Yet there is still no convincing evidence of release of nitric oxide from this tissue. Inhibition of EDRF reduces coronary blood flow (Amezcua *et al.*, 1989) and it has been demonstrated recently in isolated, perfused hearts that 5-hydroxytryptamine stimulates NO release from the coronary microvasculature, measured by coronary sinus nitrite sampling, and this is accompanied by a reduction in the time to reach peak ventricular pressure (Shah *et al.*, 1991). These preliminary data suggest that coronary microvascular endothelium is capable of releasing vasoactive factors, and are further discussed in section 1.4..

1.2.6. **Release of nitric oxide from nitrovasodilator drugs**

Organic nitrates have been used to treat angina pectoris for over a century, but only recently has their mechanism of action been established (Henderson, 1991). Nitrovasodilator drugs cause vasodilatation by being metabolised at their site of action to produce nitric oxide. Different nitrovasodilators are metabolised by different pathways (Furchgott, 1988). Porcine coronary smooth muscle cells metabolise glyceryl trinitrate (GTN) to generate nitric oxide by a plasma membrane enzyme (Chung & Fung, 1990). Sodium nitroprusside undergoes a reduction step requiring NADPH, NADH or a thiol which takes place in the microsomes (Fung *et al.*, 1991).

Nitrovasodilator drugs are a convenient source of nitric oxide for experimental purposes. It is generally assumed that they do not have much effect on myocardial contractility. The effects of nitrovasodilators on cardiovascular haemodynamics are established in both patients and in experimental models, but the same haemodynamic changes will obscure any specific action of nitrovasodilators on contractility. The isolated, functioning cardiac myocyte is thus a suitable model to study contractility in the absence of haemodynamic changes. Whether cardiac myocytes can metabolise different nitrovasodilator drugs to generate nitric oxide has not previously been demonstrated.

1.3 **ACTIONS OF NITRIC OXIDE IN MACROPHAGES, NEUTROPHILS, PLATELETS, BRAIN/NERVOUS SYSTEM AND OTHER CELLS**

1.3.1. **Action of macrophage derived nitric oxide**

As mentioned in 1.1.6., macrophages are toxic to certain tumour cells and microbes. Nitric oxide released from macrophages activated by cytokines attacks Fe-S groups of enzymes containing this nonhaem Fe subunit. Such an enzyme is ribonucleotide reductase, the rate-limiting enzyme in DNA replication (Hibbs *et al.*, 1988). Why nitric oxide affects DNA replication in only some cells is not known (Hibbs *et al.*, 1990b).

The cytotoxic and cytostatic actions of macrophages on large microorganisms closely involve nitric oxide. Inhibition of nitric oxide production impairs substantially macrophage-mediated cell killing (Radomski *et al.*, 1987; Granger *et al.*, 1986; Liew *et al.*, 1990). Likely the free radical properties of nitric oxide account for its cytotoxicity. Administration of pure nitric oxide also kills microorganisms (Liew *et al.*, 1990).

1.3.2. **Action of neutrophil derived nitric oxide**

Although neutrophils contain constitutive nitric oxide synthase, production of nitric oxide does not seem to contribute much to their cytotoxic ability (Moncada *et al.*, 1991). Stimulation of neutrophils with formyl-methionyl-leucyl-phenylalanine (FMLP) or LTB_4 increases nitric oxide production (McCall *et al.*, 1989). Nitric oxide inhibits neutrophil aggregation (McCall *et al.*, 1990) but inhibition of nitric oxide inhibits neutrophil chemotaxis (Kaplan *et al.*, 1990). The biological role of constitutive nitric oxide synthase in neutrophils remains to be elucidated.

1.3.3. **Role of nitric oxide in platelet function**

Nitric oxide elevates cGMP in platelets, inhibiting both adhesion and aggregation (Ayuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987). PGI_2 and adenosine inhibit platelet aggregation by increasing cAMP. These three autacoids act in synergy (Radomski *et al.*, 1987). The growth factor platelet derived growth factor (PDGF) is released during platelet adhesion, and nitric oxide thus has a further important role in the regulation of the mitogenic effects of platelets and PDGF. This is clear notably during percutaneous transluminal coronary angioplasty (PTCA) (Brady & Warren, 1991) when subendothelial tissues are exposed.

Endothelium derived nitric oxide has a role modulating platelet function at sites of vascular injury. Activated platelets release ADP and serotonin, both of which stimulate nearby intact endothelium to release EDRF, limiting the propagation of platelet aggregation on to healthy intima, while the absence of nitric oxide at sites of injury allows platelets to perform their function (Henderson, 1991).

1.3.4. **Action of nitric oxide in the nervous system**

Nitric oxide in brain appears to be intimately involved in modulation of amino acid neurotransmission. Emerging studies are suggesting a role for nitric oxide in long term synaptic depression and potentiation, processes thought to be central to learning and memory (Nathan, 1992). Another reaction which may involve nitric oxide is the toxicity of glutamate during ischaemic cerebral vascular events (Dawson *et al.*, 1991), which may be mediated in part by nitric oxide acting as a free radical. This suggests a possible role for inhibitors of nitric oxide or nitric oxide synthase in treatment of ischaemic stroke (Nowicki *et al.*, 1991).

In the peripheral nervous system, nitric oxide is co-released at non-adrenergic, non-cholinergic (NANC) nerve terminals, and also appears to be involved in the mechanism of penile erection (Garthwaite, 1991). Constitutive nitric oxide synthase activity has been demonstrated in the myenteric plexus of the intestine (Snyder & Bredt, 1991).

1.3.5. **Nitric oxide in other cells**

Fibroblasts produce nitric oxide when stimulated with cytokines, as do Kupffer cells in the liver, and it is likely that this is a property of all reticuloendothelial cells, and may indeed be found in many other cell types (Nathan, 1992).

1.4 **ACTIONS OF NITRIC OXIDE IN THE HEART**

1.4.1. **Sources of nitric oxide in heart**

Nitric oxide activity is found in coronary endothelium and endocardial endothelium in health. This activity may be induced in coronary vascular smooth muscle and myocardial interstitial cells in certain conditions. Demonstration of its presence and function in myocardial contractile cells forms the major part of the work described in this thesis. The pericardium remains as the only cardiac tissue not yet

known to produce nitric oxide.

The function of nitric oxide in epicardial and resistance coronary arteries is probably no different to the other vascular beds described above, *i.e.* regulation of vascular tone and maintenance of homeostasis. Evidence from animal studies has confirmed the importance of nitric oxide in the maintenance of coronary vascular tone (Kelm & Schrader, 1990). Another study of the coronary circulation in Langendorff-perfused guinea pig hearts showed that inhibition of nitric oxide synthase abolished autoregulation of coronary flow (Ueeda *et al.*, 1992).

Alteration in endothelial function may be particularly important in pathological conditions. Examples include stable atheroma, unstable angina, Syndrome X and acute myocardial infarction. These are discussed in section 1.5..

1.4.2. **Coronary microvascular endothelium**

There is much microvascular endothelium within the heart. Figure 3, (reproduced with permission from the publishers) from the Handbook of Physiology, demonstrates this. Elastomer injected into the coronary circulation fixed the vasculature and myocytes and supporting tissue were chemically dissolved, leaving the dense network of capillary vessels, as shown. These lie in close proximity to cardiac muscle, so that most myocytes are within 8 μm of their nearest capillary (Rose & Goresky, 1984; Randall, 1984). This short diffusing distance means that myocytes may be influenced by vasoactive factors produced by adjacent endothelium. The hypothesis that cardiac myocyte contraction is sensitive to endothelium derived nitric oxide thus became the basis for my first series of experiments.

Important differences exist between the endothelium lining large arteries, and endothelium within the microvasculature. For example, coronary microvessels are less sensitive than aorta to the exogenous nitrovasodilator, GTN, but relax equally to NO solution, albeit at a higher concentration (Sellke *et al.*, 1990). This implies that metabolism of organic nitrates in the coronary microvasculature is less efficient than elsewhere in the circulation, perhaps because of a relative deficiency of either the enzymes required (Chung & Fung, 1990), or available sulfhydryl groups necessary for generation of NO from GTN. NO has been shown to be a vasodilator in several

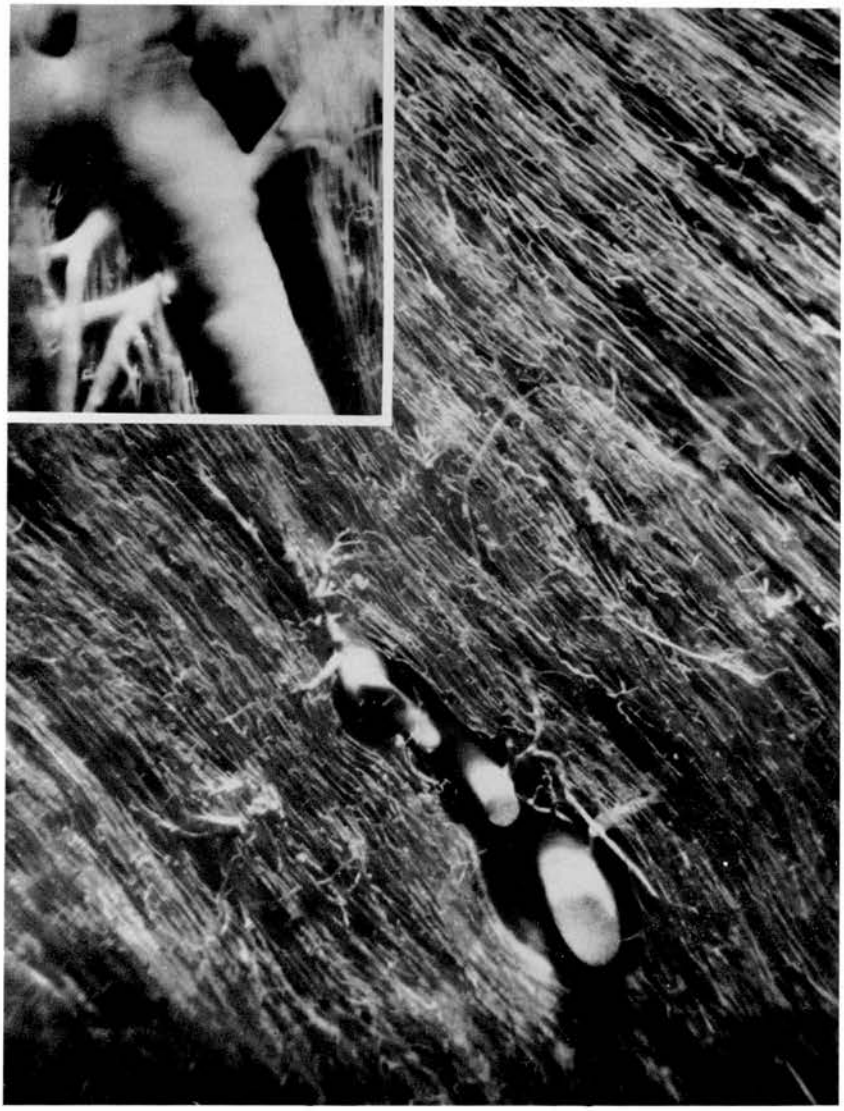


Figure 3 Canine coronary microvascular endothelium, x400.

different microvascular preparations (Warren *et al.*, 1992; Gardiner *et al.*, 1990; Ekelund & Mellander, 1990; Persson *et al.*, 1990).

Nitric oxide release from coronary microvascular endothelium has only been demonstrated indirectly. Inhibition of EDRF reduces blood flow in the coronary vascular bed as it does in others (Amezcua *et al.*, 1989). Recent studies in isolated, perfused hearts using 5-HT to stimulate nitric oxide release showed a reduction in the time to reach peak ventricular pressure. Nitric oxide release from the coronary microvasculature was shown by analysis of coronary sinus nitrite (Shah *et al.*, 1991).

The detergent Triton X-100 is used experimentally to denude papillary muscles of endocardial endothelium. In organ baths this agent can be washed quickly off the preparation. Careful ultrastructural and functional analysis has shown no damage to subendothelial structures using this method. Preliminary data have recently been presented on the effects of infusing Triton X-100 in the coronary circulation (Li *et al.*, 1992). Contractile parameters were decreased by this action, and although these data support the hypothesis that coronary microvascular endothelium influences myocardial contraction, the explanation that the effects of so powerful a toxin in the coronary circulation are purely on microvascular release of nitric oxide remains speculative.

Another study using isolated guinea pig hearts showed that intracoronary methylene blue or the nitric oxide scavenger oxyhaemoglobin both increased coronary vascular resistance and decreased cGMP production (Kelm & Schrader, 1990). Bradykinin, which releases nitric oxide from endothelium, dilated coronary resistance vessels and this was accompanied by elevation of nitrite levels in coronary sinus blood.

Although there is still no direct evidence from cultured microvascular endothelium from hearts showing release of nitric oxide more circumstantial evidence has been reported from Lewis' group, investigating further the effect of bradykinin on contractile function in intact guinea pig hearts. Perfusion with bradykinin reduced myocardial contractility, and this effect was abolished by the inhibitor of nitric oxide synthase, L-NMMA (Fort *et al.*, 1992). Another explanation is that the effect is secondary to alterations in coronary resistance vessel tone caused by bradykinin, not to a direct effect of stimulated microvasculature on cardiac myocyte contractility.

1.4.3. **Endocardial endothelium and its role in the modulation of myocardial contraction**

Until recently endocardium was considered, like vascular endothelium, not to be of much importance other than as a structural element of the heart, but with an important role in preventing thrombus formation within cardiac chambers. It lines the trabeculated inner surface of the atria and ventricles. In support of the hypothesis that important interactions exist between vascular lining cells and the myocardium, recent studies have now shown that endocardial endothelium influences adjacent myocardial cells by releasing at least two humoral factors which have opposing effects on papillary muscle contraction (Smith *et al.*, 1991). This was first described by Brutsaert and coworkers who showed that damage to the endocardial surface of isolated papillary muscle by brief exposure to Triton X-100 detergent causes a reduction of about 20% (at 2.5 mM $[Ca^{2+}]_o$) in maximum isometric tension produced by the muscle, brought about by an earlier onset of isometric relaxation, but with no effect on the velocity of contraction (Brutsaert *et al.*, 1988). This effect occurs in endocardium-intact preparations with administration of lipid-soluble analogues of cGMP, or with sodium nitroprusside and atrial natriuretic peptide (substances that increase myocardial cGMP by stimulating soluble and particulate guanylate cyclase respectively), which reduce papillary muscle contractility similar to endocardial damage (Smith *et al.*, 1991; Brutsaert *et al.*, 1988; Shah & Henderson, 1992).

From experiments using cultured endocardial cells on beads in a cascade system, endocardial endothelium appears to release both EDRF and an as yet unidentified substance, termed endocardin, which both augments contraction and prolongs relaxation (Smith *et al.*, 1991). This substance does not have the characteristics of the action of the powerful endothelial vasoconstrictor, endothelin on papillary muscle, which itself has inotropic effects. Restoration of endocardial products to endocardium-damaged papillary muscles both increases the force of isometric contraction and prolongs the relaxation phase after contraction in this system. The quantitative relationship between this as yet unknown endocardial product and endocardial EDRF is yet to be fully established, although the unidentified contraction-prolonging factor appeared to be dominant in these experiments (Shah &

Henderson, 1992).

The interpretation of the effects of endocardial products on myocardial contractility is that endocardium may influence both systolic (myocardial contraction) and diastolic function (myocardial relaxation). The predominant effects of EDRF and endocardin are on late systolic contraction *in vitro*, and this is likely to have effects on events early in diastole, *i.e.* myocardial relaxation and ventricular filling. These effects may be particularly important in conditions where myocardial contraction and relaxation are abnormal, or the heart rate is rapid and tachycardia unduly prolonged (Henderson, 1991; Shah & Henderson, 1992; Smith *et al.*, 1991; Smith *et al.*, 1992).

Exciting work from this group has just demonstrated that endothelin can be produced by endocardial endothelium, although its place in the modulation of myocardial contraction is yet to be established (AJ Shah, personal communication).

Further important work on papillary muscle function in inflammatory conditions is emerging. This is discussed in chapters 7 and 10.

1.4.4. **Nitric oxide release from perivascular nerves in heart**

Nitric oxide mediates vasomotor nerve-induced vasodilatation in skeletal muscle in rabbits (Persson *et al.*, 1990). In heart, substance P nerve fibres form part of the NANC innervation of the coronary circulation. Substance P affects coronary blood flow and vascular tone by an endothelium-dependent mechanism (see 1.5.1.), probably by diffusing from nerve terminals to endothelium to reach its receptors (Weihe *et al.*, 1981; Crossman *et al.*, 1989). There is no evidence that other NANC neurotransmitters, for example calcitonin gene-related peptide (CGRP) and neuropeptide Y, act via an endothelium-dependent mechanism.

Recently, immunohistochemical evidence of nitric oxide synthase has been found in cardiac nerve fibres and ganglion cells (Klimaschewski *et al.*, 1992). Its functional significance is not yet clear, but possible sites of nitric oxide activity include myocytes, conducting tissue and vasculature.

1.4.5. **Nitric oxide activity in myocardium**

There is a body of evidence concerning the function of nitric oxide in the coronary circulation and in endocardial endothelium. Over recent months reports of a role for nitric oxide within myocardium have begun to accumulate. Such studies require a reductionist approach, since measurement of contractile function during application of vasoactive agents to patients or whole animals is complicated by haemodynamic changes due to the vasoactive agent's vascular effects.

Evidence from studies of endocardium had shown that contractility of papillary muscle myocardium could be modified by EDRF (see 1.4.3.). Evidence that cardiac myocyte contraction is affected by nitric oxide forms the main body of the work of this thesis. At the same time biochemical evidence of nitric oxide synthase activity in myocardium has become available. This work and discussion of available evidence is presented in chapters 4-9 and chapter 11.

1.5. **NITRIC OXIDE IN PATHOLOGICAL CONDITIONS**

1.5.1. **Hypertension and atherosclerosis**

Infusion of inhibitors of nitric oxide synthase raises blood pressure in animals by blocking tonic production of nitric oxide in resistance vessels. Disturbances in endothelial function may contribute to hypertension. Some possible theories are:-

impairment of nitric oxide release from arteriolar endothelium;

abnormalities of nitric oxide agonist receptors;

altered nitric oxide diffusion because of smooth muscle hypertrophy;

alterations in cellular second messengers/cellular breakdown of cAMP and cGMP.

Endothelial responses are impaired in animal models of hypertension (Lamping & Dole, 1987; Van de Voorde & Leusen, 1986; Winquist *et al.*, 1984; De Mey & Gray, 1985; Luscher *et al.*, 1987; Otsuka *et al.*, 1988), and in patients with high blood pressure (Panza *et al.*, 1990; Linder *et al.*, 1990; Calver *et al.*, 1992). Further

studies are awaited in this key area of cardiovascular research to place these early findings in context.

1.5.2. **Atherosclerotic vascular disease**

Atherosclerosis is characterised by the development of plaques. These lesions develop within the intima, replacing healthy endothelium with a hyperplastic collection of lipid-rich material, macrophages, smooth muscle cells and fibrous tissue, usually covered by endothelium. Small lesions are universal in Western adults. Problems arise when a plaque grows gradually and becomes flow limiting, or when it suddenly enlarges following haemorrhage within the plaque or when thrombus develops on its surface, and then occludes the vessel.

Endothelium overlying stable plaques is abnormal, both in function and in ultrastructural appearance. In animals made hyperlipidaemic, endothelial responses to agents which stimulate EDRF release are diminished, while responses to endothelium-independent (*e.g.* sodium nitroprusside) vasoactive agents is intact (Coene *et al.*, 1985; Verbeuren *et al.*, 1986; Freiman *et al.*, 1986; Shimokawa *et al.*, 1988). Interestingly, in animal models of hyperlipidaemia there is increased production of oxides of nitrogen (Minor *et al.*, 1990) and this may be due to induction of inducible nitric oxide synthase in lungs (Lang *et al.*, 1993).

Endothelial dysfunction has been demonstrated in experimental models of diabetes (Henderson, 1991). Accumulating evidence points to the scavenging effect of haemoglobin as the mechanism of the cerebral vasoconstriction seen in subarachnoid haemorrhage. It may be that blood in the subarachnoid space seeps into the intimal layers of intrathecal arteries, inhibiting EDRF released from endothelium of those vessels (Henderson, 1991).

1.5.3. **Coronary artery disease**

In patients with atheromatous coronary artery disease impaired responses to endothelium dependent vasodilators occur. Acetylcholine causes dilatation of normal segments but constriction of vessels, not only at areas with severe atherosclerosis on

angiography but in vessels with "minimal" disease also (Ludmer *et al.*, 1986). Responses to glyceryl trinitrate (GTN) were unaffected. Of course for atheromatous lesions to appear on angiography means that significant disease must be present, so their findings probably represent a spectrum of impaired response, rather than two different disease processes. The endothelium-dependent vasodilating neuropeptide, substance P dilates normal coronary arteries (Crossman *et al.*, 1989). This effect is abolished or much attenuated at sites of atherosclerosis (Mathew *et al.*, 1992), supporting the hypothesis of endothelial dysfunction in atheromatous arteries.

Loss of tonic EDRF release at sites of atheroma might predispose towards focal coronary spasm. This is a possible explanation of the type of coronary hyperreactivity described by Prinzmetal (Prinzmetal *et al.*, 1959). It is tempting to speculate on the role of nitric oxide in microvascular angina, but to date no evidence exists to support such conjecture. EDRF activity in the peripheral vasculature is impaired in models of chronic heart failure, and in the coronary arteries of patients with dilated cardiomyopathy (Henderson, 1991). Thus, there is much evidence indicating the importance of nitric oxide in atherosclerotic disease, and much more is forthcoming.

Recently, the effects of intracoronary administration of L-NMMA in patients with normal coronary arteries has been examined. Basal distal left anterior descending (LAD) coronary artery diameter and basal coronary blood flow were slightly reduced, and acetylcholine-induced distal dilatation was abolished. There was no effect on the increase in coronary blood flow caused by acetylcholine (LeFroy *et al.*, 1992). This study provides further information about a tonic role of nitric oxide in epicardial coronary arteries. The behaviour of the microcirculation was not addressed in this study.

1.5.4. **Restenosis after percutaneous transluminal coronary angioplasty (PTCA)**

Endothelium has a key role in the development of restenosis after PTCA. Most evidence available points to the regulation of smooth muscle cell growth by endothelial-derived heparin proteoaminoglycans. The necessary loss of endothelium during PTCA exposes sub endothelial tissue to platelet- and other growth factors.

Smooth muscle then undergoes the phenotypic and hyperplastic change, accompanied by remodelling of the extracellular matrix proteins, characteristic of vascular repair after PTCA. In about a third to a half of cases this remodelling becomes excessive and leads to restenosis (Brady & Warren, 1991). Interestingly, nitric oxide inhibits DNA synthesis in vascular smooth muscle *in vitro* (Nakaki *et al.*, 1990). Thus loss of endothelium at PTCA removes this additional regulator of smooth muscle proliferation.

Drug therapy to prevent restenosis has so far not succeeded. Most of the compounds used affect coagulation or vessel tone, rather than mimicking endothelial control of cell division. Nitrovasodilators are used to inhibit coronary spasm immediately following PTCA. Evidence that they inhibit restenosis in patients is lacking. Whether nitric oxide regulates vascular smooth muscle growth in patients has yet to be described.

1.5.5. **Endotoxic shock**

Septicaemia and accompanying septic shock account for a substantial number of hospital deaths, despite appropriate antibiotic and supportive therapy. In the USA, an estimated 100,000 patients die from sepsis in hospital each year (Parrillo *et al.*, 1990). One of the characteristic features of septic shock is profound hypotension caused by a decrease in peripheral vascular resistance. This hypotension is unusually resistant to both volume replacement and vasoconstrictor agents. In the earliest stages of septic shock stroke volume and cardiac output are maintained or even increased; later, ventricular dilatation develops with a reduction in ejection fraction (MacLean *et al.*, 1967; Parker *et al.*, 1984; Ellrodt *et al.*, 1985). If patients survive, ventricular size and function return to normal as the infection is controlled and circulatory function restored.

Gram-negative bacteria account for about 30% of cases of septic shock (Bone *et al.*, 1987). Bloodborne infection liberates endotoxin, the lipopolysaccharide component of the bacterial cell wall, into the circulation. Endotoxin and the organisms themselves activate host defence and inflammatory systems, including the complement, kinin and coagulation cascades, the interleukins, tumour necrosis factor

(TNF) and other endogenous mediators of inflammation, leukocytes and platelets which together generate the acute inflammatory response to bacteraemia. Although many inflammatory mediators are themselves vasoactive, recent work has shown that the hypotension of septic shock is mediated by important changes within the vascular muscle cells themselves. The key finding of the work in this thesis is the demonstration that the myocardial depression is caused by similar changes.

1.5.6. **Abnormalities of nitric oxide production in endotoxic shock**

In endotoxic shock the presence of disseminated foreign antigen, together with the inflammatory response, causes nitric oxide synthase to be induced in many other cell types which do not normally express this enzyme, including hepatocytes, fibroblasts and vascular smooth muscle (Moncada *et al.*, 1991; Nathan, 1992). Subsequent production of nitric oxide leads not only to haemodynamic instability, but also to widespread production of nitric oxide-based free radicals which have the potential to cause considerable damage to tissues. Evidence from clinical studies supports this. Patients with endotoxic shock (Ochoa *et al.*, 1991), and cancer patients receiving interleukin-2 (IL-2) chemotherapy, a cytokine which activates other endogenous cytokines to induce nitric oxide synthase (Hibbs *et al.*, 1992), excrete high levels of nitric oxide metabolites.

Vascular smooth muscle is itself not a source of nitric oxide in health. In endotoxic shock production of nitric oxide occurs within the muscle layer of the vessel wall, and this causes excessive vasodilatation and hence a reduction in peripheral vascular resistance. Analogues of the substrate L-arginine have been developed which act as substrate inhibitors of nitric oxide synthase. These cause systemic vasoconstriction and a pressor response in healthy animals by inhibiting constitutive nitric oxide production by the endothelium. Some studies have shown a fall in cardiac output with these agents as well.(Klabunde & Ritger, 1991; Kilbourn *et al.*, 1990; Aisaka *et al.*, 1989) In healthy human volunteers local administration of these drugs into the brachial artery causes a 50% reduction in forearm blood flow (Vallance *et al.*, 1989).

In animals with experimental endotoxic shock inhibitors of nitric oxide synthase reverse hypotension, but also cause a sustained increase in systemic vascular resistance and at higher doses a decrease in cardiac output.(Klabunde & Ritger, 1991; Nava *et al.*, 1991; Wright *et al.*, 1992) Whether this fall in cardiac output is secondary to the rise in vascular resistance, or to an adverse effect of nitric oxide synthase inhibitors on cardiac contractility, was not established by these studies.

A recent study in dogs points to the activation of ATP-sensitive K^+ channels in vascular smooth muscle by acidosis as a further mechanism of vasodilatation in experimental endotoxic shock.(Landry & Oliver, 1992) This has not yet been explored in patients.

1.5.7. **Cardiac failure in endotoxic shock**

Global deterioration of myocardial contractile function in endotoxic shock has been well established by clinical and radionuclide studies (MacLean *et al.*, 1967; Parker *et al.*, 1984; Ellrodt *et al.*, 1985). A study in normal volunteers showed reversible depression of left ventricular function following administration of purified endotoxin, in addition to the expected reduction in systemic vascular resistance, measured both by radionuclide scanning and by echocardiography (Suffredini *et al.*, 1989). Until recently, the cause of this myocardial depression was considered to be a direct effect of endotoxin or an inflammatory mediator on myocardial tissue, to which coronary hypoperfusion might contribute (Parrillo *et al.*, 1990). The existence of a specific circulating myocardial depressant substance in endotoxic shock has been postulated, although not proven (Reilly *et al.*, 1989). Although coronary perfusion abnormalities occur in patients with coexisting cardiac or coronary disease and may account for segmental abnormalities of left ventricular function, in patients with global myocardial impairment and endotoxic shock the loss of function cannot be explained by changes in coronary flow (Parrillo *et al.*, 1990; Ellrodt *et al.*, 1985). As in the peripheral vasculature, it is likely that multiple factors exist which depress cardiac function in endotoxic shock. However, there may be a common pathway for such mediators to impair myocardial contraction.

In the same way that overproduction of nitric oxide in the peripheral

vasculature accounts for the vasodilatation and loss of vascular control in endotoxic shock, the notion that overproduction of nitric oxide within cardiac muscle contributes to impaired function is the key issue addressed in this thesis.

1.6. MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

1.6.1. Natural history of injury induced by ischaemia and reperfusion

The presence of inflammatory cells in infarcted myocardium was first described more than 50 years ago (Mallory *et al.*, 1939). The importance of inflammatory processes in successful healing after myocardial infarction was revealed more recently when methylprednisolone given to post infarction patients caused a substantial increase in both infarct size and deaths from ventricular rupture due to poor scar formation (Roberts *et al.*, 1976). This indicated that inflammatory mechanisms are necessary for optimal repair after infarction. However, such inflammatory processes may also damage nearby healthy or reversibly injured myocardium.

Following irreversible coronary occlusion the earliest manifestations of necrosis can be demonstrated in myocyte organelles and contractile elements by electron microscopy within one hour (Schaper *et al.*, 1992), although gross and microscopic changes do not become apparent until 6-8 hours after the onset of ischaemia (Anderson, 1985). The features of coagulative necrosis then appear: after about 8 hours infiltration by neutrophils occurs, with the development of a zone of granulation tissue at the margin of the infarct (Levine, 1929; Mallory *et al.*, 1939). The infarct heals by digestion of necrotic tissue by macrophages with subsequent scar formation, and the infarct zone is replaced gradually by fibrous tissue. Large numbers of neutrophils accumulate in the dead myocardium (Go *et al.*, 1988), although whether they are involved in killing myocytes alive at the time of reperfusion is uncertain.

After periods of up to three hours of ischaemia (Chatelain *et al.*, 1987) reperfused myocardium is characterised by the early accumulation of neutrophils, firstly within the blood vessels and then later within the injured myocardium (Sommers & Jennings, 1964). There is recovery of some of the injured myocytes and herein lies the central controversy of recovery after a short period of ischemic injury: *does the process of reperfusion with delivery of acute inflammatory components cause irreversible injury of myocytes which might have regained normal function, or does*

reperfusion merely hasten the death of cells irretrievably injured? This question remains unresolved, but in the next sections, evidence is presented that interference with some of these inflammatory processes reduces ultimate infarct size.

1.6.2. **Pathogenesis of inflammatory injury after myocardial ischaemia**

Neutrophils are important mediators of injury in ischaemia-reperfusion damage. Depletion of circulating neutrophils by means of cytotoxic agents (Mullane *et al.*, 1984) anti-neutrophil antibodies (Romson *et al.*, 1983) or leukocyte filters (Litt *et al.*, 1989) all reduce myocardial infarct size in experimental models. The protective action of agents such as ibuprofen and prostacyclin (Romson *et al.*, 1982; Simpson *et al.*, 1987b) is also associated with a reduction in neutrophil accumulation.

The chemoattractants, complement fragment C5a, and the eicosanoid, leukotriene B_4 (LTB_4) both mediate neutrophil damage within the heart. Complement activation occurs following episodes of myocardial ischaemia (Pinckard *et al.*, 1975; Langlois & Gawryl, 1988). Complement fragments are found in ischaemic myocardium (Maroko *et al.*, 1978; Schafer *et al.*, 1986; Rossen *et al.*, 1985).

Cobra venom factor, which depletes circulating complement, reduces infarct size and neutrophil accumulation (Maclean *et al.*, 1978; Maroko *et al.*, 1978), and also reduces neutrophil chemoattractant activity of coronary sinus blood following myocardial ischaemia (Hartmann *et al.*, 1977). Neutrophil chemoattractant activity in postischaemic cardiac lymph can be neutralised by antibodies to C5a (Entman *et al.*, 1991). Soluble complement receptor 1 (sCR1), which inhibits complement activation by binding C3 and C5 convertases, also reduces infarct size and neutrophil infiltration (Weisman *et al.*, 1990).

LTB_4 is produced by activated neutrophils and is detected in infarcted myocardium (Sasaki *et al.*, 1988). Inhibitors of the 5-lipoxygenase enzyme (which catalyses synthesis of LTB_4) reduce infarct size and neutrophil accumulation (Mullane *et al.*, 1987; Sasaki *et al.*, 1988). Some of these agents possess other properties such as free radical scavenging activity. LTB_4 receptor antagonists are now available, although early results are mixed (Karasawa *et al.*, 1991; Hahn *et al.*, 1990).

Platelet activating factor (PAF) is another mediator of inflammation within the heart. PAF antagonists reduce myocardial infarct size in experimental models (Stahl *et al.*, 1988; Montrucchio *et al.*, 1990; Chakrabarty *et al.*, 1992), although mechanisms other than inhibition of neutrophil accumulation may contribute (Williams *et al.*, 1990).

Mechanism of tissue damage by neutrophils

Neutrophils cause tissue injury by releasing two main groups of toxic substances: reactive oxidising chemicals and proteolytic enzymes. Membrane bound NADPH oxidase enzyme catalyses the production of superoxide anions, hydrogen peroxide and hydroxyl radicals. These radicals are able to react with a number of biological substrates themselves, but another product of oxidative metabolism, hypochlorous acid (HOCl), may be of even greater importance (Harrison & Schultz, 1976; Weiss, 1989). HOCl and oxygen free radicals cause cellular injury by oxidising membrane phospholipids, thereby increasing fluidity and permeability of the membrane and reducing its integrity. They also augment the activity of neutrophil proteolytic enzymes, which degrade components of the extracellular matrix.

Myocardial damage by oxygen free radicals

Since the 1970s it has been accepted that reoxygenation of ischaemic myocardium may be associated with immediate and extensive ultrastructural damage, and the damage is caused, at least in part, by free oxygen radical species derived from the administered oxygenated medium (Hearse, 1977). Neutrophils also generate these reactive radicals although to what extent oxygenated blood, neutrophils and the reperfused tissue itself each contributes to the amount of free radical damage has not been quantified. The role of oxygen free radicals in myocardial infarction has been the subject of a large number of studies using agents which can scavenge these radical species. Of 32 studies of interventions to reduce free radical damage in animal models of myocardial injury, 16 showed benefit and 16 did not (Reimer *et al.*, 1989). Although the scavenging agents catalase and superoxide dismutase can neutralise oxygen free radicals in the extracellular space, they are large molecules and do not readily enter cardiac myocytes (Downey & Yellon, 1992). Yet within myocytes free

radicals generated during ischaemia-reperfusion cause damage to contractile proteins (Shlafer *et al.*, 1987; Turner *et al.*, 1991). This may explain the modest protection afforded by scavenging agents in the published studies.

Recently, monitoring of neutrophil-mediated cardiac myocyte oxidative damage at the cellular level has become possible using dichlorofluorescein (an intracellular indicator of oxidation). Neither catalase nor superoxide dismutase had a protective effect in these studies (Entman *et al.*, 1992). In contrast, desferrioxamine and dimethylurea, both of which can enter cells, protected myocytes from neutrophil injury. Some of the protective effect of dimethylthiourea is thought to be due to its ability to inhibit Na^+-Ca^{2+} exchange (Ziegelstein *et al.*, 1992). There is accumulating evidence for the importance of this process in reperfusion injury. There is impairment of Na^+-Ca^{2+} exchange, Na^+-K^+-ATPase and Na^+-H^+ exchange in ischaemia (Tani & Neely, 1989; Kitakaze *et al.*, 1988) which may facilitate Ca^{2+} entry into myocytes. Disturbance of intracellular calcium homeostasis may be the central feature of ischaemic injury (Steenbergen *et al.*, 1987b; Snyder & Bredt, 1991).

Effects of ischaemia on coronary microvasculature

One of the consequences of ischaemia is injury to the coronary microvasculature itself. Successful reperfusion of the large coronary arteries may not always be accompanied by restoration of blood flow within the area of injured myocardium. This has been termed the "no-reflow" phenomenon (Kloner *et al.*, 1974), and appears to follow endothelial injury after periods of ischaemia greater than 60-90 minutes. Plugging of the microvessels by neutrophils during reperfusion contributes to the no-reflow phenomenon, and is likely mediated both by neutrophil adhesion to damaged endothelium, together with neutrophils wedging in the microvessels. Such physical trapping occurs because activated neutrophils are less deformable, and hence their ability to squeeze through capillary lumina is impaired (Worthen *et al.*, 1989). I addressed this in an early study using a microvasculature model of endothelial cells cultured on beads (Brady *et al.*, 1990) (see section 9.2).

In the clinical situation, the no-reflow phenomenon occurs when restoration of patency is achieved by thrombolysis or angioplasty, yet reperfusion of myocardium

in the ischaemic area remains impaired. Such impairment of myocardial perfusion is associated with a worse outcome, measured in terms of left ventricular ejection fraction, than patients in whom microvascular and hence myocardial tissue reperfusion is achieved (Ito *et al.*, 1992).

Ultrastructural studies suggest that endothelium tolerates hypoxia better than cardiac myocytes, since zones of no-reflow are found only within areas of myocardial necrosis (Piper *et al.*, 1990; Kloner *et al.*, 1974). However there is accumulating evidence that important alterations of endothelial function may occur much earlier in ischaemia, without evidence of morphological damage.

Basal release of nitric oxide is diminished following ischaemia-reperfusion, and this is associated with neutrophil adherence to coronary endothelium (Ma *et al.*, 1993). Administration of pharmacological agents which mimic endothelial products, including nitric oxide (Johnson *et al.*, 1991), Iloprost (a stable analogue of prostacyclin) (Simpson *et al.*, 1987a) and adenosine (Olafsson *et al.*, 1987) reduce experimental infarct size, probably both by replenishing protective endogenous autacoids lost during the ischaemic episode and also by inhibiting neutrophil-endothelial interactions. Such interventions may become clinically important, since limiting the no-reflow phenomenon may allow better myocardial perfusion following epicardial coronary artery occlusion-reperfusion. Unfortunately these agents all have potent vasoactive effects which may limit their usefulness. In an early study in which I was involved, Iloprost had beneficial effects on platelet aggregation in patients undergoing cardiopulmonary bypass, but hypotension precluded widespread application (Blauth *et al.*, 1987). Other analogues of these agents may be more useful.

1.6.3. **Myocardial response to acute ischaemia - cellular events and intercellular signalling**

Neutrophils have the potential to damage myocardium by their release of toxic products. Whether such toxins are liberated in a random fashion by activated neutrophils within an area of injured myocardium, or whether they are targeted towards injured myocytes in particular has not yet been determined. However,

emerging evidence suggests that injured cardiac myocytes may signal their own destruction by activating such mechanisms at their cell surface.

Neutrophil-endothelial adhesion

For neutrophils to reach the interstitium and then the myocytes they have to adhere to and then traverse the endothelial barrier. At sites of inflammation neutrophils roll along endothelium by engaging and disengaging to the endothelial cells by adhesive interactions involving the neutrophil glycoprotein L-selectin (formerly termed LAM-1, Mel-14, LECAM-1, Leu-8) (Lawrence & Springer, 1991). Tethering of neutrophils at sites of inflammation occurs via the expression of the endothelial glycoprotein P-selectin (formerly GMP-140, PADGEM, CD62) which appears on cell surfaces within minutes of endothelial activation by inflammatory mediators (Geng *et al.*, 1990). The rapid expression of P-selectin suggests that it may mediate early neutrophil adhesion to activated or injured endothelium, since it is expressed from intracellular or cell membrane stores without the need for synthesis of new proteins (Geng *et al.*, 1990). P-selectin interacts with its carbohydrate ligand CD15 on the neutrophil (Larsen *et al.*, 1990). It appears that the neutrophils undergo an early change in the expression of their adhesion molecule presentation, with L-selectin exhibiting a rapid increase in affinity followed by down regulation of its expression and the activation of the CD18 adhesion glycoprotein complex (Vedder & Harlan, 1988; Nourshargh *et al.*, 1989).

Migration of neutrophils across the endothelial layer follows adhesion, and requires contact between the neutrophil integrin CD18 complex and the endothelial adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) (Smith *et al.*, 1988). Activated endothelium synthesises and expresses the adhesion glycoproteins, ICAM-1, and also E-selectin (formerly endothelial-leucocyte adhesion molecule-1). ICAM-1 is present constitutively on endothelium at low levels but its expression is increased substantially by inflammatory mediators (Colditz & Movat, 1984). Monoclonal antibodies to the CD18 antigen have been shown to be very effective in reducing neutrophil infiltration into ischaemic-reperfused myocardium (Williams *et al.*, 1990) and in reducing myocardial infarct size (Seewaldt-Becker *et al.*, 1990). In the latter study a monoclonal antibody to ICAM-1 was also protective.

E-selectin only appears on the endothelium after stimulation (Bevilacqua *et al.*, 1989) and requires *de novo* protein synthesis. The corresponding ligand for E-selectin on the neutrophil has been identified recently as another carbohydrate, sialylated Lewis x tetrasaccharide (Lowe *et al.*, 1990). Both E-selectin and ICAM-1 mediate neutrophil adhesion to and migration through activated endothelium. It may be envisaged that after early initiation of neutrophil adhesion to ischaemic-reperfused endothelium by endothelial expression of P-selectin, further neutrophil-endothelial adhesion to damaged myocardium may be promoted by the ICAM-1/CD18 and E-selectin/sialylated Lewis x tetrasaccharide mediated interactions. This hypothesis is supported by current research. Very recently, increased production of mRNA for both P- and E-selectin has been demonstrated in the endothelium of blood vessels from the injured segments of ischaemic-reperfused hearts (DC Anderson, personal communication).

Neutrophil-cardiac myocyte interactions

Once neutrophils have reached the extravascular space they can directly attack cardiac myocytes. Whether this damage is directed specifically towards previously injured myocytes is not known, but current research indicates that activated myocytes appear to exhibit signals which activate neutrophil wound repair mechanisms. Recent studies indicate that cardiac myocytes as well as endothelial cells express ICAM-1 in response to activation by inflammatory mediators (Smith C.W. *et al.*, 1991). Neutrophils bind to ICAM-1 on activated myocytes by a process involving their adhesion molecule complex CD18 (Entman *et al.*, 1990), and this receptor-mediated adhesion appears to be the main mechanism of neutrophil adherence to cardiac myocytes. Furthermore, mRNA for ICAM-1 can be induced in both endothelial cells and cardiac myocytes by post-ischaemic cardiac lymph (Youker *et al.*, 1992), and recent data suggest that direct neutrophil-mediated injury of cardiac myocytes requires adhesion by the CD18/ICAM-1 mechanism (Entman *et al.*, 1991). However, expression of ICAM-1 by myocytes requires *de novo* protein synthesis (Entman *et al.*, 1990). Since it has been shown that complement activation products are present on the surface of myocytes following ischaemia, one mechanism of early neutrophil attachment might be via a CD11b/CD18 (complement receptor 3) interaction with iC3b. Another

possible early mechanism is attachment of neutrophils to a PAF-like molecule which is expressed on the surface of myocytes soon after their injury, without the need for protein synthesis (CW Smith, personal communication).

It is possible therefore that administration of monoclonal antibodies to CD18 or ICAM-1 are able to confer protection in models of myocardial ischaemia by inhibiting direct neutrophil-cardiac myocyte interactions as well as by inhibiting neutrophil migration through the endothelium (Simpson *et al.*, 1990; Simpson *et al.*, 1988; Ma *et al.*, 1992), although these experiments have not always shown benefit (Tanaka *et al.*, 1993). These recently published and ongoing studies provide exciting new evidence that injured myocardium plays an active role in the inflammatory response to ischaemic injury.

1.7. CENTRAL HYPOTHESES OF THIS THESIS

(i) **Nitric oxide modifies contractile function of cardiac ventricular myocytes.**

Nitric oxide affects cellular function of many different cell types. My hypothesis is that nitric oxide modifies the major function of cardiac myocytes, *i.e.* their contractile function.

(ii) **Cardiac myocytes metabolise nitrovasodilators to generate nitric oxide.**

Nitrovasodilators act by being metabolised at their site of action to generate nitric oxide. Glyceryl trinitrate and isosorbide dinitrate require an enzyme but sodium nitroprusside requires only a reduction step. My hypothesis is that cardiac myocytes are able to generate nitric oxide from nitrovasodilators and modify their contractility.

(iii) **Nitric oxide from coronary microvascular endothelium modifies contractility of adjacent cardiac myocytes.**

Nitric oxide derived from endothelium modulates tone in adjacent vascular smooth muscle. Within the myocardium, the coronary microcirculation lies in close proximity to cardiac muscle. My hypothesis is that within the heart also, nitric oxide from microvascular endothelium modifies cardiac myocyte contraction.

(iv) **Nitric oxide production within cardiac myocytes impairs their contractility in endotoxic shock.**

Profound hypotension is a feature characteristic of endotoxic shock, caused by induction of nitric oxide synthase within the smooth muscle of the vascular wall. Cardiac function is also depressed in endotoxic shock. My hypothesis is that, much like vascular smooth muscle, cardiac myocytes themselves produce nitric oxide in this situation and depress their own contractility.

(v) **Nitric oxide production within cardiac myocytes contributes to the impaired contractility of postischaemic myocardial stunning.**

Myocardial contractility is reversibly depressed following a short period of

ischaemia. My hypothesis is that activity of constitutive nitric oxide synthase within cardiac myocytes contributes to this impairment of contraction.

(vi) **Nitric oxide modulates cardiac myocyte contractility by reducing peak Ca^{2+} concentration during contraction.**

The results of studies testing hypotheses i-iv showed that nitric oxide attenuates cardiac myocyte contraction. Attenuated contraction is caused either by a reduction in $[Ca^{2+}]_i$ during the cardiac action potential, or by a change in the sensitivity of the myocyte contractile apparatus to calcium. My hypothesis is that nitric oxide reduces the amount of calcium available in the myocyte during contraction, leading to diminished contractility.

(vii) **Neutrophils exert direct action on cardiac myocytes to impair their contractility.**

Neutrophils are important in myocardial ischaemia. Their role in wound repair after infarction is established. But neutrophils may also cause early damage during the reperfusion period after ischaemia. Many studies examine alterations in infarct size as a consequence of interference with neutrophil function during a period of ischaemia-reperfusion, mainly using *in vivo* models. But whether neutrophils damage myocardium because of microvascular obstruction and resulting local ischaemia, or because of a direct action on myocytes, cannot be determined from these studies. My hypothesis is that adhesion of activated neutrophils to myocytes directly impairs contractility, and ultimately leads to myocyte death.

CHAPTER 2: METHODS

2.1.1. Isolation of guinea-pig cardiac ventricular myocytes

The method of isolation of cardiac myocytes from laboratory animals by enzymatic digestion of myocardium has been established in the National Heart and Lung Institute (and previously the Cardiothoracic Institute) for nine years (Harding

Figure 4

et al., 1988; Harding *et al.*, 1990; Vescovo *et al.*, 1989). This method yields healthy functioning cardiac myocytes, an example of which is shown in Figure 4. I used this method in all of the experiments using adult guinea-pig myocytes, except those involving cells from ischaemic-reperfused hearts, where I conceived of and developed a modified method of isolation (section 3.6).

To isolate ventricular myocytes from healthy animals, heparin 1000 U *i.p.* was injected 15 min before sacrifice. The guinea-pig was killed by cervical dislocation and the heart quickly removed and placed in 50 ml cold Krebs-Henseleit solution (containing 119.1 mM NaCl, 1.0 mM $CaCl_2$, 4.7 mM KCl, 0.94 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.5 mM glucose, and previously bubbled with 95%0_2/5%CO_2 at 4°C) with 1000 U heparin added. The heart was dissected free of excess tissue and mounted by the ascending aorta on a Langendorff perfusion apparatus. The aorta was perfused retrogradely and coronary flow established. Coronary perfusion with physiological Krebs-Henseleit solution at 37°C and 20 ml/min continued for 5 min to allow the heart to recover. The perfusate was then changed to a low calcium solution (containing 120 mM NaCl; 5.4 mM KCl; 15 μM $CaCl_2$; 5 mM $MgSO_4$; 5 mM pyruvate; 20 mM taurine; 10 mM HEPES, 5 mM nitrilotriacetic acid (NTA) and 20 mM glucose) which facilitated breakdown of intercellular junctions. Asystole usually occurred within 20-30 s. The low calcium perfusion continued for 4 min 30 s and was followed by 2 min perfusion of enzyme solution (containing 120 mM NaCl, 5.4 mM KCl, 200 μM $CaCl_2$, 5 mM $MgSO_4$, 5 mM pyruvate, 20 mM taurine, 10 mM HEPES and 20 mM glucose) containing 4 I.U. protease (type XXIV, bacterial, Sigma). This was followed by collagenase-hyaluronidase solution (same enzyme solution with 0.6 mg/ml hyaluronidase, type I-S, bovine, Sigma; and collagenase 0.3 mg/ml, Class II, Worthington, New Jersey, U.S.A.), recirculated for 10 min.

The heart was then removed from the Langendorff apparatus, the apex and right ventricle excised and discarded and the left ventricle minced gently with scissors. This tissue was swirled in two successive digests of collagenase-hyaluronidase solution, each for 5 min. The supernatant from each was centrifuged and the myocyte pellet resuspended in the 200 μM calcium solution for use in experiments.

A third digest was often performed, swirling the myocardial tissue in a high K^+ solution (containing 20 mM NaCl, 85 mM KCl, 30 mM KH_2SO_4, 5 mM $MgSO_4$, 20 mM taurine, 0.5 mM NTA and 20 mM glucose). If the myocyte yields from the first and second digests were reasonable, they were preferred.

This method of isolation of myocytes yielded reliably functioning cells. The

first digest often contained cellular debris from the enzymatic digestion, but the second was comparatively pure. Healthy myocytes are rectangular; terminally contracted ones are round. The "rod" to "round" ratio of morphologically sound cells to apparently irreversibly injured cells was established visually. Preparations used for experiments usually contained at least 60-70% rod shaped cells.

2.1.2. **Definition of myocyte viability**

Isolated myocytes were defined as healthy and used in experiments if they fulfilled the following criteria:

(i) Cells were rod shaped.
(ii) Cells were without sarcolemmal blebs (a sign of irreversible injury).
(iii) Cells did not exhibit spontaneous contractions (except atrial cells).
(iv) Cells that displayed a variable baseline contraction to electrical stimulation at 2mM Ca^{2+} were rejected.
(v) Sarcomere length was within the range 1.67-2.05 μm. Average sarcomere length was calculated from the number of striations crossing the screen at high magnification in most of the experiments. Cells were excluded if sarcomere length was shorter than 1.67 μm, since this is an indication of cell injury.

2.1.3. **Purification of cardiac myocyte preparation**

To improve the ratio of morphologically functioning myocytes I tried purification of the pellet by a short centrifuge period in a carbohydrate gradient. This technique is used widely to isolate leucocytes from whole blood. Such a gradient has been described in early work on isolation of cardiac myocytes (Maisch, 1981). I attempted to use the published method to improve myocyte yield for experiments involving myocyte-neutrophil interactions (chapter 10). This method required lengthy high-speed centrifugation of Percoll to produce a gradient of continuously variable density. I simplified this and used just two different densities of Percoll, *i.e.*, a discontinuous gradient, the interface of which would match the point on the variable

gradient at which healthy myocytes were found.

In practice, rounded up, terminally contracted myocytes have a density only fractionally less than healthy cells, according to their respective positions on the Percoll gradient. In fact, they ought to be *more* dense, since they lose fluid contents during the rounding-up process. An explanation of this may be that rod-shaped myocytes are more streamlined than rounded cells, and so travel through carbohydrate more easily.

While endothelial cells and other debris could be excluded, differentiation between healthy and rounded myocytes was not readily reproducible using a Percoll gradient. Thus, purification of myocytes was not pursued.

I examined the behaviour of myocytes in a fluorescence activated cell sorting (FACS) flow cytometry cell analyser (Becton-Dickinson). I was interested in the expression of adhesion molecules on cardiac myocytes, using fluorescent-labelled antibodies to ICAM-1 as an indicator. Cardiac myocytes have significant intrinsic autofluorescence and labelling them with either of the fluorescent dyes fluorescein or rhodamine did not alter the fluorescence of test suspensions of cells by a substantial degree. Thus differences between labelled and non-labelled cells would not be determined. Workers at Baylor, Houston, Texas had similarly failed to use FACS cell sorting to study expression of adhesion molecules on myocytes (ML Entman, personal communication). Recently however, they have successfully used direct observation of fluorescence and also fluorometric measurements to detect neutrophil-mediated myocyte injury (Entman *et al.*, 1992).

2.1.4. **Isolation of human ventricular myocytes**

Human ventricular myocytes were used in some experiments. A block of ventricular myocardium was obtained from the recipient heart of a patient receiving a transplant, usually for dilated cardiomyopathy, sometimes for end stage ischaemic heart disease. Tissue obtained was transported to the laboratory in cold cardioplegic solution (containing Na 131 mM; K^+ 16 mM; Ca^{2+} 2 mM; Cl^- 111 mM; lactate 29 mM; procaine 1 mM) and then minced using an array of razor blades. The tissue obtained was incubated at 35°C in 25-30 ml low calcium solution (containing 120

mM NaCl; 5.4 mM KCl; 1-2 μM $CaCl_2$; 5 mM $MgSO_4$; 5 mM pyruvate; 20 mM taurine; 10 mM HEPES, 5 mM NTA and 20 mM glucose) for 3x4 min. Tissue was then transferred to a similar solution (without NTA) containing 4 U/ml type XXIV protease (Sigma). Ca^{2+} was made up to 30 μM and the solution swirled gently at 35°C for 45 min. This process was repeated twice using the solution of the same composition, except 400 IU/ml collagenase (Worthington) was added instead of protease. Dispersed cells were strained and centrifuged in the same way as guinea pig ventricular myocytes, described in 2.1.1..

2.2 MEASUREMENT OF CARDIAC MYOCYTE CONTRACTILITY

2.2.1. Design of myocyte cell bath

Adult cardiac ventricular myocytes do not contract spontaneously *in vitro*, unlike atrial myocytes and neonatal myocytes. Most of the studies in this thesis

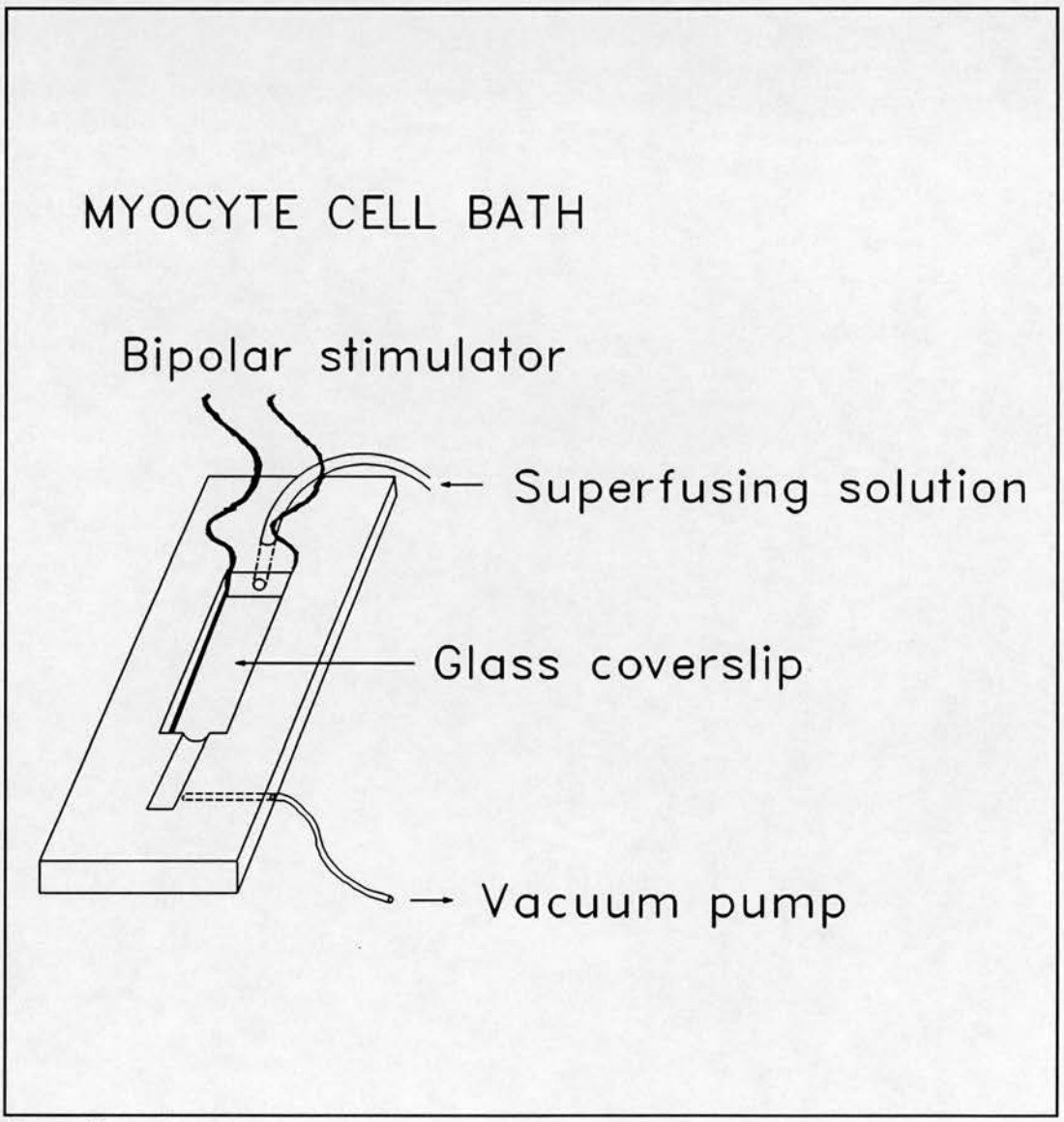


Figure 5

examine modulations of myocyte contractility, thus a method of stimulating the cells to contract is required.

A 22 mm glass coverslip was fitted under a chamber 10 x 4 x 6 mm in

dimensions cut into a perspex block (Figure 5). Isolated myocytes were placed in the chamber and attached spontaneously to the glass. After 4 min to allow attachment, cells were superfused with Krebs-Henseleit physiological solution at 2 ml/min. The cell bath was drained by a channel drilled into the end opposite the superfusing cannula. The position of the channel maintained the volume of solution in the bath at 200 μl. The cell bath had a platinum wire along each side to enable stimulation of myocytes.

2.2.2. **Control of temperature of cell bath**

The superfusing solution was warmed by a circumferential heater around the cannula at the entrance to the cell bath. A thermocouple next to the heater was connected to a feedback control on the heater control unit, and temperature was also monitored by a second thermocouple within the bath itself. For most of the experiments when superfusing flow was constantly running, this system could maintain bath temperature accurately within 32 ± 0.5°C.

For the experiments using coculture of myocytes with endothelium flow was halted for 5 min to allow accumulation of endothelial-derived factors within the cell bath. I showed in control experiments that changes in temperature affect markedly myocyte contractility (see 4.1.4.). Since stopping flow causes bath temperature to fall, an indwelling thermistor connected directly to a pen recorder was introduced, so that fluctuations in bath temperature could be determined readily. A moveable 60 W external heating source was placed near the bath. Altering its positioning accurately controlled solution temperature in the cell bath, so that paired recordings of baseline contraction, preceding or following the effects of stimulation of endothelium on myocyte contractility, could be made at the same temperature.

2.2.3. **Electrical stimulator**

Myocytes were stimulated electrically to contract using platinum electrodes which ran the length of the cell bath (Figure 5). The wires were connected to a stimulator which delivered a bipolar square wave stimulus. Voltage, pulse-width and frequency could be modified as desired. The threshold voltage required to cause contraction of myocytes was measured, and the stimulator set at 1.5x this level. This usually meant stimulation at approximately 30 V amplitude, 0.5 ms pulse-width. Frequency was set at 0.5 Hz, since early experiments in the department had shown cell survival to be optimal at this rate.

2.2.4. **Detection of myocyte contraction**

The cell bath was placed on the stage of a Zeiss 1M phase-contrast inverted microscope (Figure 6). Part of the light was diverted to a Newvicon video camera (Ikegami) side mounted on the microscope. The image was displayed on a high grade 9" monochrome monitor (Ikegami). The magnification factor on the screen was calibrated using a grid of known size in the cell bath. A myocyte image was displayed on the screen and the camera rotated until the long axis of the cell was vertical. The images were stored on conventional VHS video tape and could be analysed later if desired.

An edge detection device (SP 144, HVS Image Analyzing, Kingston, Surrey) was connected between the camera and the monitor. This device transforms grey into black or white, and a variable control allows adjustment of the grey level on the untransformed image at which the cutoff between black and white occurs. The level is adjusted until the imaged cell appears as the main white object on the screen. A rectangular window is placed over the cell. Within this window changes of contrast between black and white are detected. As the myocyte contracts, the number of horizontal white lines which make up its image is reduced, and this is detected by the window. The amplitude and rate of change of amplitude (*i.e.* velocity of contraction and relaxation) are thus determined, and the information converted on a digital-analogue converter built into the SP144 edge detection device and printed on a two

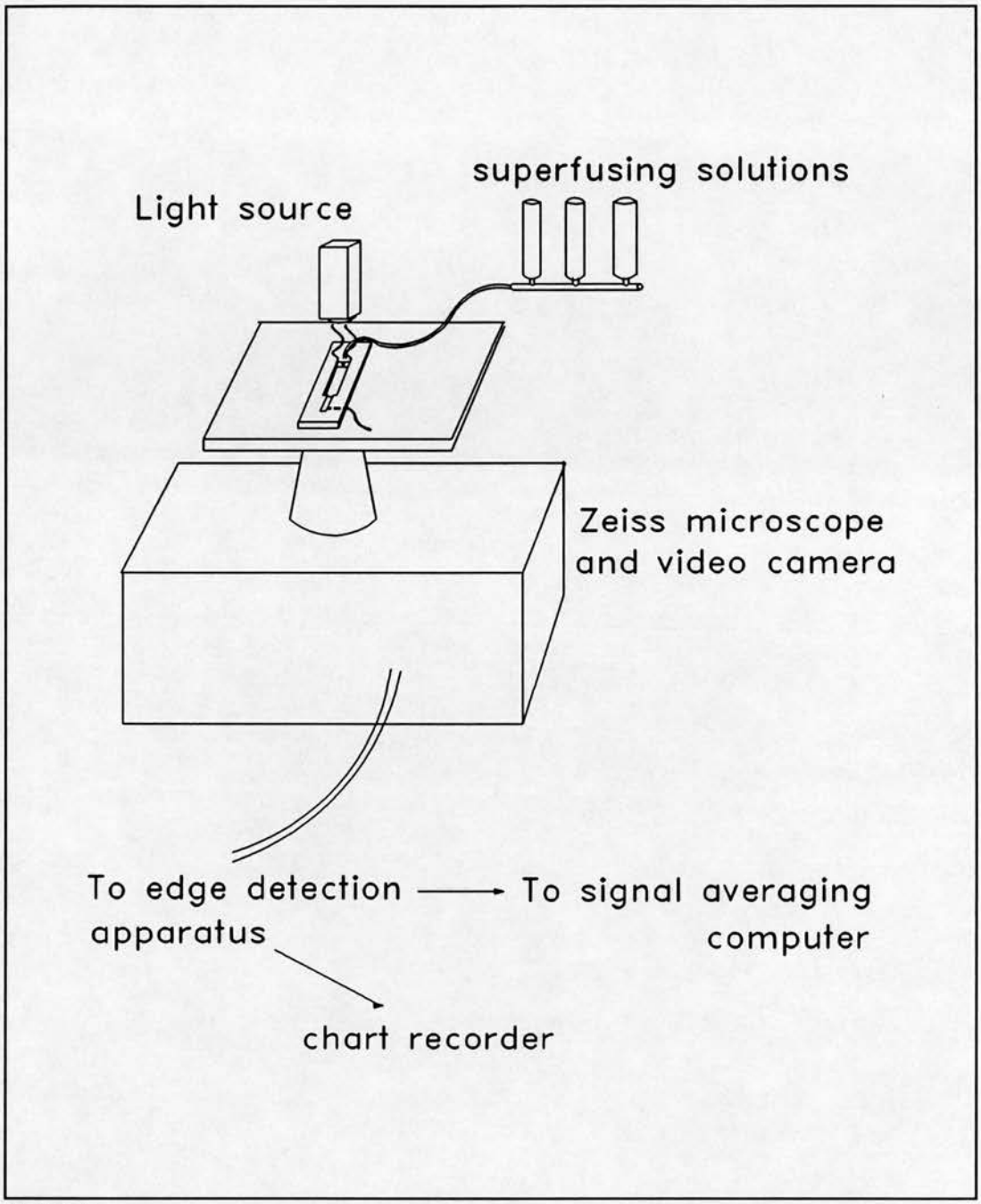


Figure 6

channel pen recorder.

The edge detection system had a time resolution of 20 ms, and a spatial resolution of 1 in 256, which allowed typically 10 to 15 sampling points within a single contraction and relaxation. Digital information could also be diverted to a computer. A program was written for acquiring and handling such data and signal-averaging of six consecutive contractions was performed to measure time to peak contraction and time from peak contraction to 90% relaxation (termed: "relaxation

time").

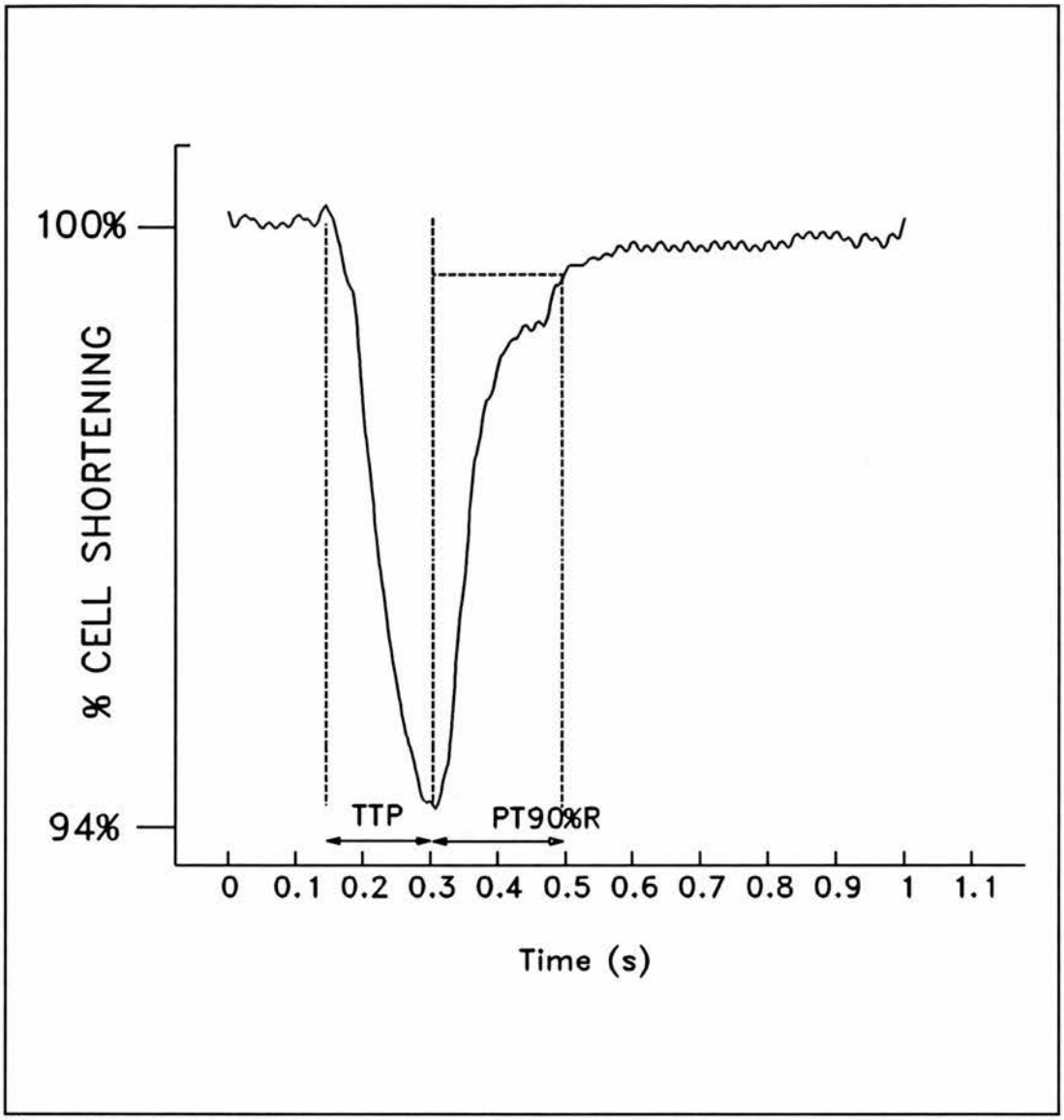


Figure 7 Representative computer-averaged trace of contraction and relaxation of a single cardiac myocyte. Measurement of time to peak contraction, and time from peak contraction to 90% of diastolic length.

Figure 7 illustrates a representative computer-drawn trace from which time dependent variables are measured.

2.2.5. **Measurement of myocyte contraction amplitude**

The myocyte image was maximised on the monitor using the largest possible lens that allowed the whole cell to be viewed. This enabled the edge detection device to operate at its greatest sensitivity and generate the largest displacement of pen on recording paper. The displacement of the pen reflected the amplitude of contraction, and this value was converted into a percentage of the diastolic length of the cell according to the formula:

$$\text{Contraction amplitude (as percentage of resting cell length)} = \frac{\text{pen displacement (mm)} \times 0.459^{*}}{\text{resting length of cell on monitor}} \times 100$$

* 0.459 = conversion factor to match the monitor image with the paper tracing.

Thus, data from each experiment was calculated as percentage changes of resting (or diastolic) length of the cell. This allowed pooling of data from different cells and comparison of data.

2.3 **Endothelial cell culture**

Culture of bovine aortic endothelium was performed without enzymatic digestion as described by J.B. Warren (Warren, 1990a), under his supervision. Once endothelial culture was established the passaging and changing of growth medium was performed by a technician. I conceived of the idea for endothelium-myocyte primary

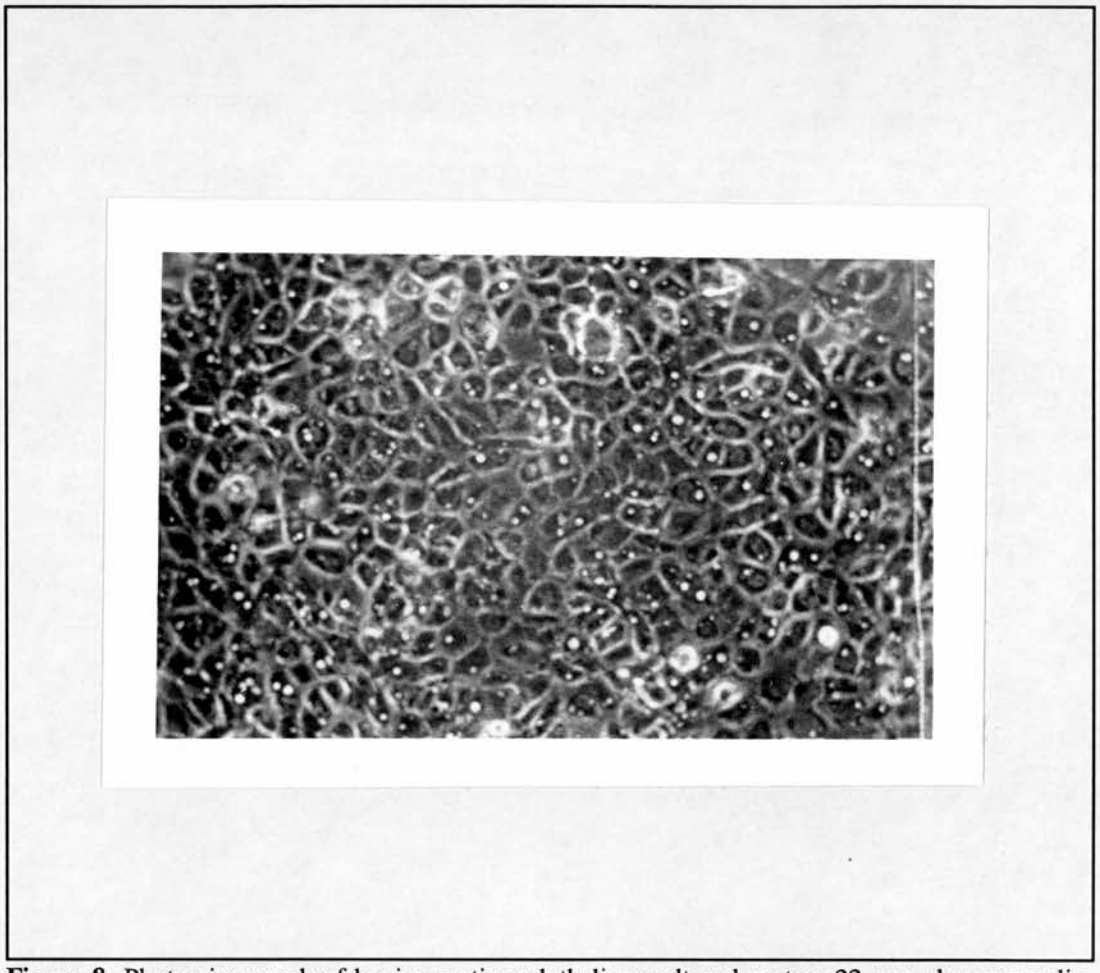


Figure 8 Photomicrograph of bovine aortic endothelium cultured on to a 22 mm glass coverslip, x 200.

coculture and then developed the method of culturing endothelium on to the 22 mm glass slides used in the inverted microscope, shown in Figure 8.

To culture endothelium, I obtained fresh bovine aortas from the abattoir. A 10 cm length of aorta was rinsed in three consecutive pots of 125 ml sterile phosphate buffered saline containing calcium and magnesium with penicillin 200,000 IU/l,

streptomycin 200 mg/l and gentamicin 100 mg/l. The vessel was cut along its length and the intimal surface scraped gently with a scalpel, rinsing the accumulating endothelial cells from the blade into a centrifuge tube containing medium. The cells were centrifuged and the supernatant discarded. The resuspended pellet was seeded into a T-25 flask.

Figure 9 Photomicrograph of primary coculture of guinea pig cardiac ventricular myocytes with bovine aortic endothelium, x 200.

Cells were cultured in a standard medium established in the laboratory. It consisted of: 500 ml Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland) with 4.5 g/l glucose but without sodium pyruvate; 50 ml fetal calf serum; 1 mg thymidine; 1/10 vial insulin/transferrin/sodium selenite (ITS) supplement (Sigma); penicillin 25,000 U; streptomycin 25 mg; and glutamine 250 mg. Contaminating fibroblasts were eliminated by mechanical passaging. Cells used in the experiments had undergone no more than 5-6 passages. Once pure cultures of bovine aortic endothelial cells were obtained, cells were cultured in 30 mm diameter multiwell

plates into which sterilised 22 mm glass coverslips had been placed. These coverslips fitted the myocyte cell bath. The endothelium grew to confluence in 2-3 days and the coverslips were ready for use.

2.4 **Endothelium and cardiac myocytes in primary coculture.**

Once the endothelial cells were confluent on 22 mm coverslips, they were ready for use in experiments. A coverslip was mounted in the cell bath and superfused with 2 mM Ca^{2+} Krebs-Henseleit solution at 32°C. A droplet of myocyte solution was placed in the bath and superfusing flow turned off for 5 min to allow the myocytes to adhere to endothelium. Endothelial cells grew only on the luminal side of the coverslips, so that viewing of myocytes through the endothelial layer was possible.

I was concerned about satisfactory adhesion of guinea-pig cardiac myocytes to bovine aortic endothelium; fortunately, the myocytes adhered to the endothelial cells, despite the presence of superfusing flow. Whether the myocytes actually were adherent to the glass via pseudopodia-like extensions between endothelial cells, or whether they were attached directly to confluent endothelium could not be determined, although the adhesion itself is an interesting phenomenon. Whatever the mechanism, the result was a primary coculture of two cell types in apposition, so that endothelial influences on myocyte contraction could be examined, as shown in Figure 9.

Baseline characteristics of the contraction of myocytes sited on endothelium were determined. Basal contractions to electrical stimulation were identical to those of myocytes adherent to glass (see Chapter 6.1.3.), hence quiescent endothelium has no effect on myocyte contractility. The effect of pharmacological activation of endothelium could then be studied.

2.5 **Endotoxic shock model**

To study the effects of endotoxaemia on cardiac myocyte function, I developed a model of endotoxic shock. Guinea-pigs were injected with a large dose of endotoxin (lipopolysaccharide, *E. coli* serotype 055:B5; Sigma). 4 mg/kg lipopolysaccharide was injected intraperitoneally, 4 h before sacrifice. All animals receiving this treatment were constitutionally upset and moved sluggishly in their cage, although blood pressure was not measured. Animals were killed and cardiac myocytes prepared as detailed in 3.1.1.. In every case cardiac myocyte contractility was substantially decreased.

In some animals, an intravenous injection of dexamethasone 4 mg/kg (David Bull, Warwick, UK) was given into an ear vein 1 h before injection of endotoxin. In controls, corticosteroid was administered 5 h before sacrifice, without endotoxin.

2.6 **Isolation and preparation of myocytes from ischaemic-reperfused hearts**

The cause of myocardial stunning is not known. To address this at the cellular level I developed modifications of the myocyte preparation protocol. Studies *in vivo* have shown that a period of ischaemia of about 20 min reliably induced myocardial dysfunction lasting approximately 1 h (Heyndrickx *et al.*, 1975). In my first attempt to recreate stunning I introduced a period of first 15, then 20 min zero coronary flow during the first stage of the Langendorff preparation, illustrated in Figure 35. I used Krebs-Henseleit solution with 1.3 mM Ca^{2+} to maintain buffer ionised calcium at normal levels during the stabilisation period before zero flow was introduced. During the ischaemia the heart was kept at 35°C by a water jacket. Hearts continued to beat under these conditions for < 15 min, although arrhythmias were usually evident after a few minutes of zero coronary flow. After the ischaemic period coronary perfusion was resumed with low Ca^{2+} solution and the myocyte preparation followed in the usual way, described in 2.1.1..

The behaviour of myocytes from early experiments was variable, although contraction often looked normal and the yield surprisingly good, frequently better than myocytes produced from hearts with no ischaemic episodes. The notion was

entertained that a period of ischaemia was actually good for the cells, in that in some way it preconditioned them against forthcoming chemical insults by enzymes.

It occurred to me that epicardial myocytes might obtain oxygen from room air, and that I was examining a heterogeneous population of cells. I addressed this in subsequent experiments by dissecting the interventricular septum and using only this tissue in the digest stages of the method. This ensured that only truly ischaemic myocytes were isolated, and epicardial ventricular myocardium was discarded.

Myocytes generated by this method still looked histologically normal and I introduced another modification to try to achieve stunning of myocytes, with less variability of contractility. Intracellular [Ca^{2+}] is elevated in ischaemic myocardium. Reperfusion with low Ca^{2+} solution protects myocardium after an ischaemic episode (Kusuoka *et al.*, 1987). Cardiac myocyte hypercontracture following reperfusion after hypoxia is characterised by a sudden further rise in $[Ca^{2+}]_i$(Josephson *et al.*, 1991). But intracellular [Ca^{2+}] is only elevated in stunned reperfused myocardium during the first few minutes following restoration of flow (Bolli, 1992), while mechanical dysfunction is prolonged. There is impairment of Na^+-Ca^{2+} exchange, Na^+-K^+-ATPase and Na^+-H^+ exchange in ischaemia (Tani & Neely, 1989; Kitakaze *et al.*, 1988) which may facilitate Ca^{2+} entry into myocytes. To further approach conditions of coronary reperfusion I introduced a short period of reperfusion with oxygenated 1.3 mM Ca^{2+} Krebs-Henseleit buffer before the low calcium solution. The results of these experiments are discussed in chapter 8.

2.7 PREPARATION OF NEUTROPHILS

2.7.1. Isolation of circulating rabbit neutrophils

New Zealand white rabbits of either sex were anaesthetized with pentobarbitone (1 mg/kg i.v.). Carotid blood was collected into falcon tubes, 9 ml per tube, each containing 1 ml acid citrate dextrose (160 mM disodium hydrogen citrate and 0.28 M glucose in sterile water) and 5 ml of 1:10 diluted acid citrate dextrose (in saline). A further 13 ml blood was collected into trisodium citrate (final concentration 0.35%) for the preparation of citrated plasma. Neutrophil preparation was carried out in a laminar flow cabinet using filtered sterile solutions and sterile pastettes and tubes.

Most of the red blood cells were removed by addition of 15 ml hydroxymethyl starch (Hespan 3% final concentration) to each tube, followed by facilitated sedimentation (centrifuging for 15 min at 25 x gravity, 20°C). The resulting leucocyte-rich plasma was centrifuged at 380 x g for 7 min. The platelet-rich plasma supernatant was removed and centrifuged twice (1952 x g, followed by 2470 x g, both at 20°C to produce platelet poor plasma (PPP). The leucocyte pellets were gently resuspended in a small volume of citrated plasma.

Neutrophils were separated from other leucocytes and remaining red blood cells using a two layer discontinuous Percoll-plasma gradient. 3 ml of 50% Percoll in plasma was carefully layered on to 3 ml 68% Percoll-plasma. Leucocyte suspension was layered on the surface and centrifuged (400 x g; 20 min, 20°C). Neutrophils were harvested from the interface of the two Percoll-plasma layers and washed twice in Ca^{2+} free Hank's solution (containing NaCl 80 g/L, KCl 4 g/L, KH_2PO_4 0.6 g/L, $Na_2HPO_4.7H_2O$ g/L, D-glucose 10 g/L, Phenol red 0.01 g/L). Cells were counted on a haemocytometer. Final concentrations were made up to 1-10x10^6/ml.

2.7.2. **Isolation of circulating human neutrophils**

In some experiments the action of human neutrophils on guinea pig ventricular myocytes was studied. Human neutrophils were also prepared for studies of neutrophil adherence to endothelium in cell columns (see section 9.2., Brady *et al.*, 1990), and some early neutrophil-guinea pig myocyte work. Human neutrophils were isolated in a manner similar to rabbit circulating neutrophils.

2.7.3. **Isolation of guinea pig peritoneal neutrophils**

There was a species problem with neutrophil-myocyte studies. Isolation of guinea pig ventricular myocytes was well established, but guinea pigs are difficult experimental animals from which to isolate neutrophils reliably from blood. I used human neutrophils and guinea pig myocytes together in the first experiments, but the species difference would always make results difficult to explain. For a time I set up a Langendorff system and made rabbit ventricular myocytes, but there were difficulties in trying to generate myocytes in one laboratory and neutrophils in another.

The answer was to use guinea pig elicited neutrophils. Injection of 10 ml 0.2% glycogen into the peritoneal cavity provokes an acute inflammatory reaction. 4h after injection animals were killed with an overdose of anaesthetic and peritoneal lavage performed. Up to 7-8 ml peritoneal fluid was obtained. This was centrifuged twice in Hank's buffer without calcium and the pellet resuspended. >90% of cells obtained were neutrophils on staining. Thus although these were not wholly quiescent cells they were readily obtainable and species problems avoided.

2.8. STATISTICAL TESTS

All values are expressed as mean±SEM, except where stated. Statistical differences between baseline and stimulated endothelium- or nitrovasodilator-influenced contraction were tested using Student's two-tailed t-test for paired data or one-way analysis of variance (ANOVA). The dose-dependence of nitrovasodilator effects was assessed using regression analysis. Statistical differences between contraction of control myocytes and myocytes from endotoxin-treated animals were tested using Student's two-tailed t-test for unpaired data. Changes between baseline contraction and contraction of cells from endotoxin treated animals under different pharmacological conditions were tested using Student's two-tailed t-test for paired data.

CHAPTER 3: VALIDATION OF EXPERIMENTAL DESIGN

3.1.1. **Definition of cardiac myocyte contractility**

Myocardial contractility, or inotropism, can be defined as the rate or extent of myocardial shortening. It is useful to regard a change in contractility as "...an alteration in cardiac performance that is independent of changes resulting from variations in preload or afterload," (Braunwald *et al.*, 1988). True changes in contractility are difficult to determine *in vivo* because of haemodynamic alterations which precede or follow interventions. Thus the isolated contracting cardiac myocyte is a good model in which to examine contractility in conditions where shortening may be modified.

Contractility is dependent on $[Ca^{2+}]_o$ in the superfusing solution used in the experiment. Physiological ionised $[Ca^{2+}]_o$ is approximately 1.3 mM. Contraction amplitude of guinea pig cardiac myocytes *in vitro* at 1 mM $[Ca^{2+}]_o$ is $2.3\pm1.0\%$ (mean$\pm$SD, n=132. Personal communication, LA Brown, PhD thesis 1992 London University) of their resting (or diastolic) length. For greater accuracy in measuring changes in contraction amplitude I used a concentration of 2 mM ionised calcium to elevate baseline contractility, to $5.3\pm0.3\%$ (mean$\pm$SEM, n=32) of resting length. This is analogous to the preconstriction of aortic rings using K^+ or prostaglandins in superfusion experiments where vascular tone is being examined. In experiments on human cells I maintained $[Ca^{2+}]_o$ at 1.3 mM as baseline contraction amplitude was acceptable.

In most experiments an intervention was performed on a single myocyte, and changes in contractility were compared to the mean of baseline recordings made before and after the intervention. This avoided the problem of myocyte fatigue. When lengthy dose response protocols are followed, basal contractility gradually declines when cells are stimulated for periods >2 h. None of my studies used prolonged protocols. Only in the experiments examining methylene blue did I not compare myocyte responses before and after exposure to the agent. The reasons for this are discussed in chapters 4 and 7.

3.1.2. **Effects of calcium and isoprenaline on cardiac myocyte contraction**

A maximum inotropic response, measured as maximal contraction amplitude achieved, is obtained by elevating $[Ca^{2+}]_o$ or exposing the myocyte to the ß-adrenoceptor agonist, isoprenaline. I intended to compare maximum amplitude of cells from stunned hearts to that of normal cells. This is discussed further in chapter 8. The effects of elevating $[Ca^{2+}]_o$ and isoprenaline on contraction amplitude are illustrated below. Figure 10 shows a chart recording used to measure contraction amplitude in response to elevated $[Ca^{2+}]_o$ and isoprenaline.

Figure 11 below shows computer-generated traces used to measure the time dependent variables, time to peak contraction, and relaxation time. Exposure of this myocyte to 8 mM calcium from a basal $[Ca^{2+}]_o$ of 1.3 mM increased contraction amplitude from 4.3% to 9.3% of resting length. Exposure of the same cell to 10^{-9}M isoprenaline increased amplitude to a level equally as high, although it is usually the case that isoprenaline has not quite the inotropic effect of maximum tolerated $[Ca^{2+}]_o$ (quoted value is 79.5±1.7% of contraction amplitude at Ca^{2+}_{max}, n=46, Wynne *et al.*, 1993). Interestingly, ß-adrenoceptor agonists frequently cause the characteristic "dicrotic" notch in the relaxation phase of the contraction, visible as a double twitch on direct visualisation.

3.1.3. **Effect of cell bath temperature on cardiac myocyte contractility**

Biochemical processes involving enzymes are temperature dependent. While this is a truism, different laboratories examine aspects of myocyte function at quite different temperatures. Experiments in the laboratory to which I was attached were performed at 32°C. To examine the role of nitric oxide synthase I wished to maintain bath temperature within tightly controlled limits. This is readily achieved when continuous superfusion is performed, with a thermocouple-controlled heating coil around the tubing. For the experiments where myocyte behaviour was examined in the presence of endothelium it was necessary to halt buffer flow to allow accumulation of endothelial products. Cell bath temperature falls rapidly when flow is halted so an external 60W heating lamp was placed near the cell bath.

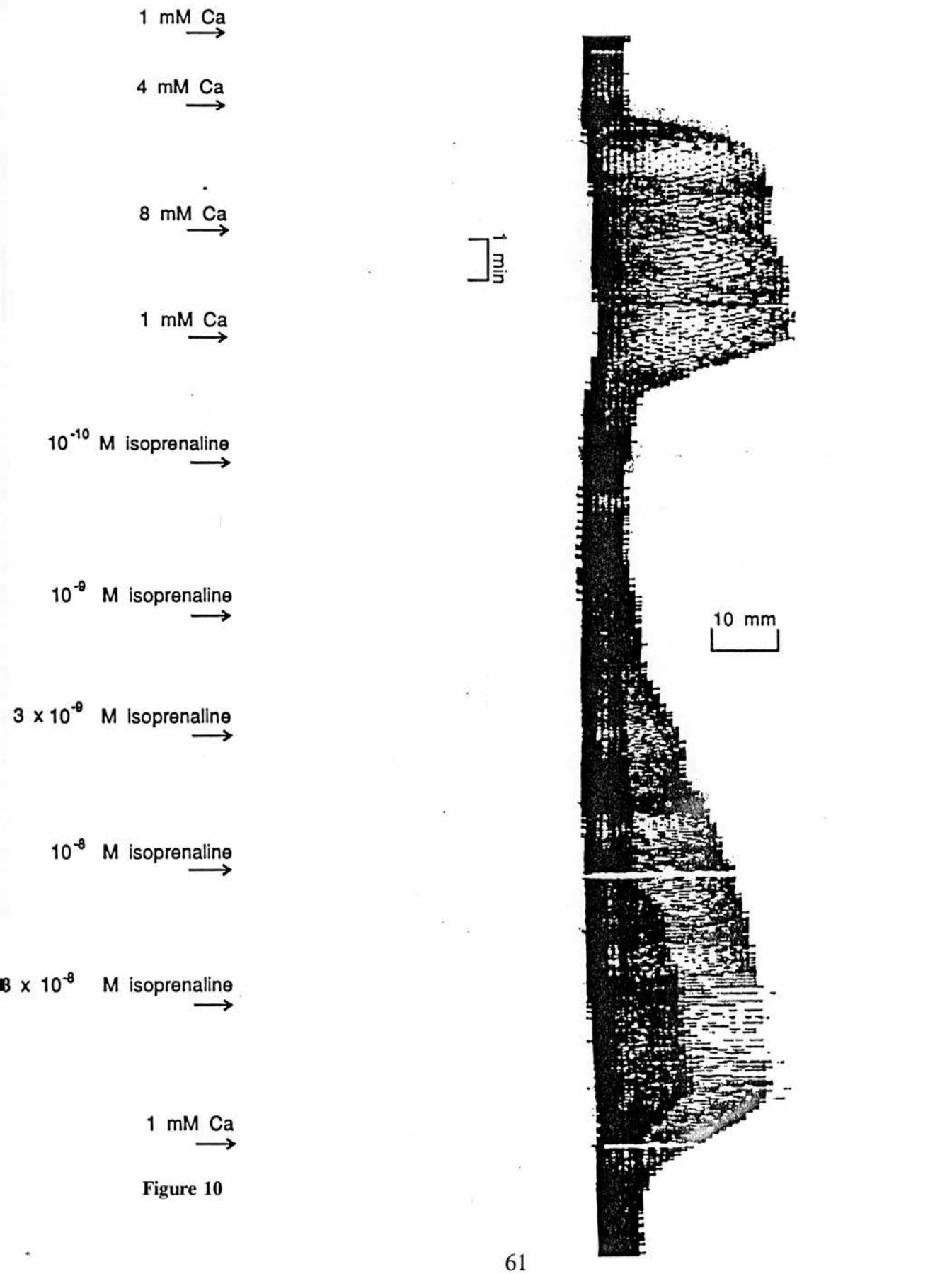

1 mM Ca
4 mM Ca
8 mM Ca
1 mM Ca
10-10 M isoprenaline
10-9 M isoprenaline
3 x 10-9 M isoprenaline
10-8 M isoprenaline
3 x 10-8 M isoprenaline
1 mM Ca
1 min
10 mm

Figure 10

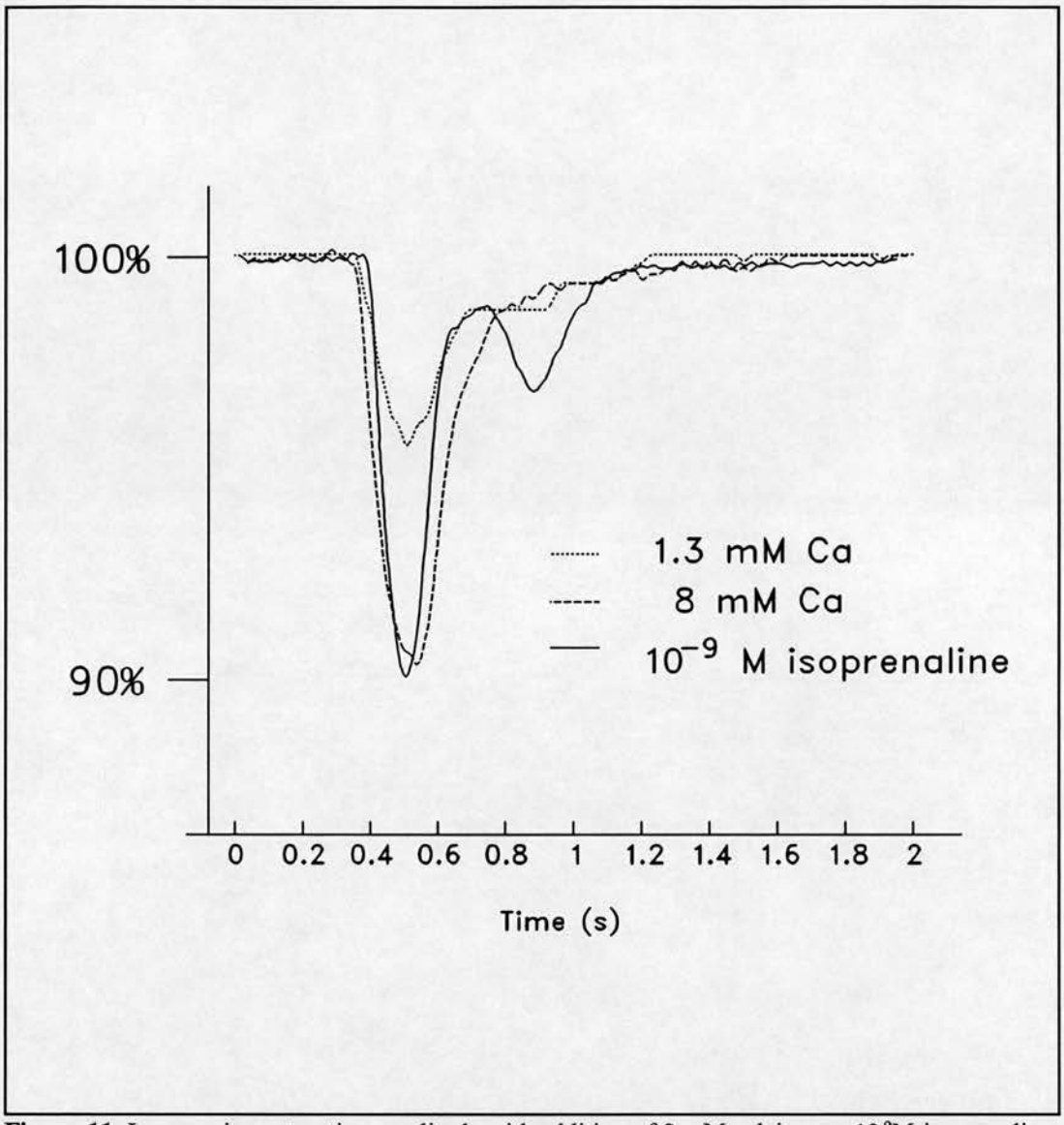


Figure 11 Increase in contraction amplitude with addition of 8 mM calcium or 10^{-9}M isoprenaline. Note the characteristic "dicrotic" notch during the relaxation phase of the isoprenaline contraction.

Bath temperature was monitored with a direct reading glass-tipped thermistor inside the cell bath, calibrated before every experiment, and the 60W heat source adjusted to maintain bath temperature at 32°C.

I studied the effects of changes in bath temperature over the range 28-36°C on normal myocytes at 2 mM $[Ca^{2+}]_o$. Amplitude of contraction was higher at lower temperatures, but velocity of contraction and relaxation was slower. These data are shown below, in Figures 12-15. A change of +1.0°C from 32°C reduced contraction amplitude by 5±0.5% (mean±SEM, n=8), and similarly, a change of -1°C increased contraction by 5±0.5% (n=7).

The explanation of this finding possibly relates to the kinetics of cellular calcium handling. At lower temperatures the rate of opening and closing of calcium channels will be reduced, perhaps allowing more calcium to enter the cytoplasm.

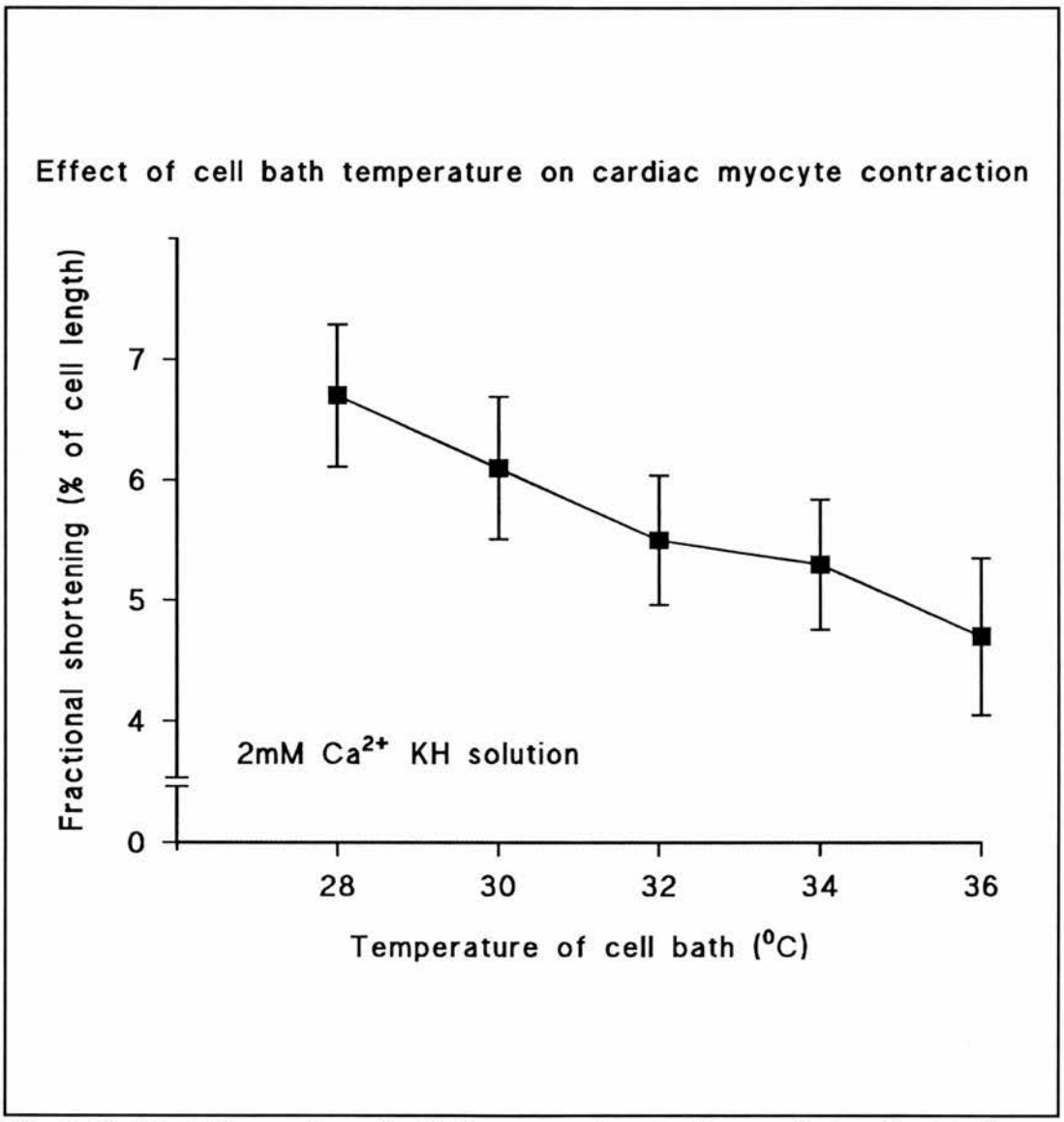


Figure 12 Effect of temperature of cell bath on myocyte contraction amplitude. Absolute changes in percentage shortening shown, n=8.

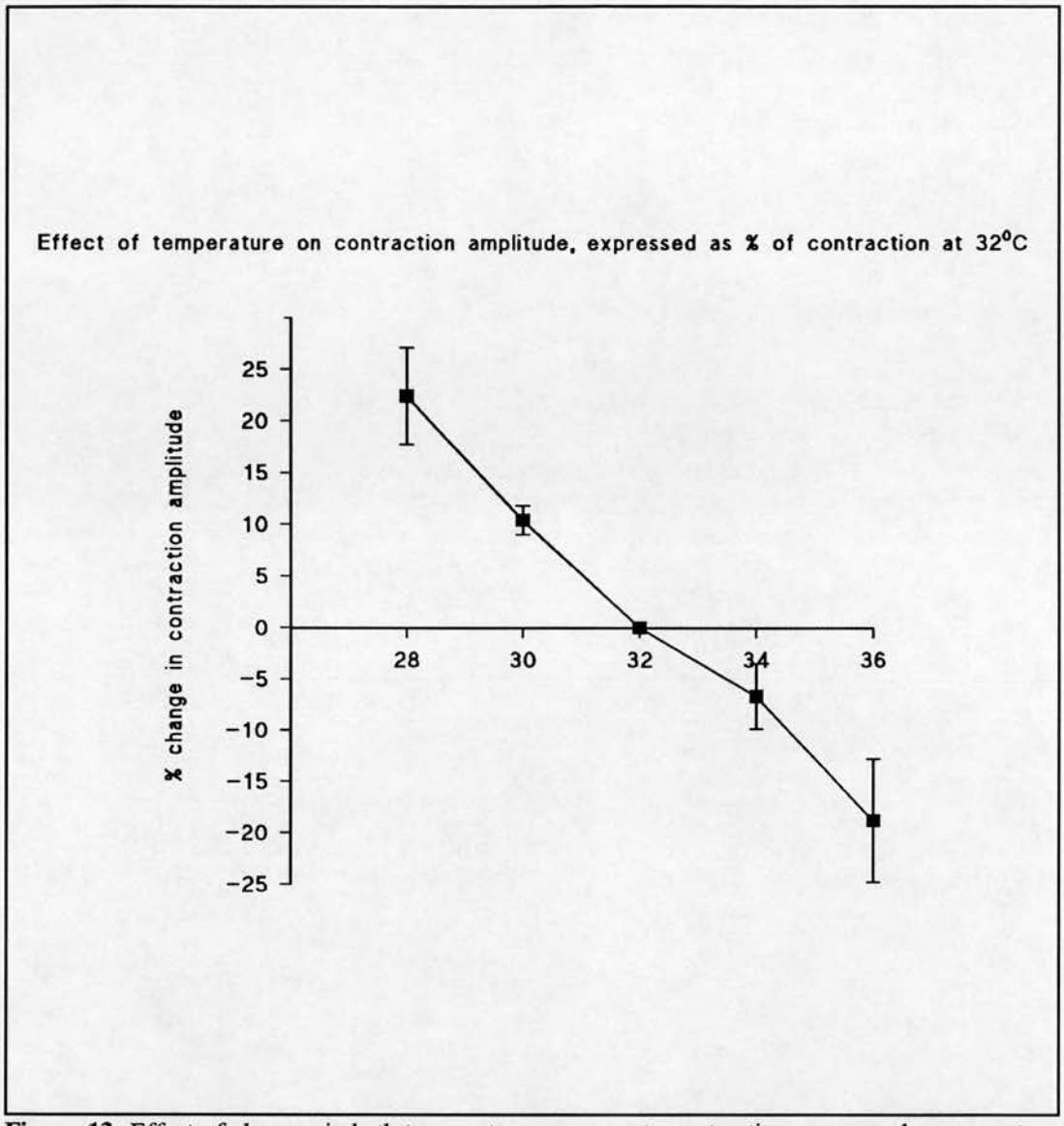


Figure 13 Effect of changes in bath temperature on myocyte contraction, expressed as percentage change from 32°C baseline, n=8.

3.1.4. **Comparison of myocyte contraction on endothelium compared to glass**

Most of the experiments in this thesis were performed on cardiac myocytes sited on glass coverslips. To establish that baseline contractility was no different when myocytes were sited on quiescent, unstimulated endothelium I compared basal contractility on the two surfaces.

Contraction amplitude of myocytes adherent to quiescent endothelium ($5.3\pm0.6\%$ of resting length, n=12) was not different to myocytes adherent to glass coverslips ($5.3\pm0.3\%$, n=32). There was no difference in time to peak contraction

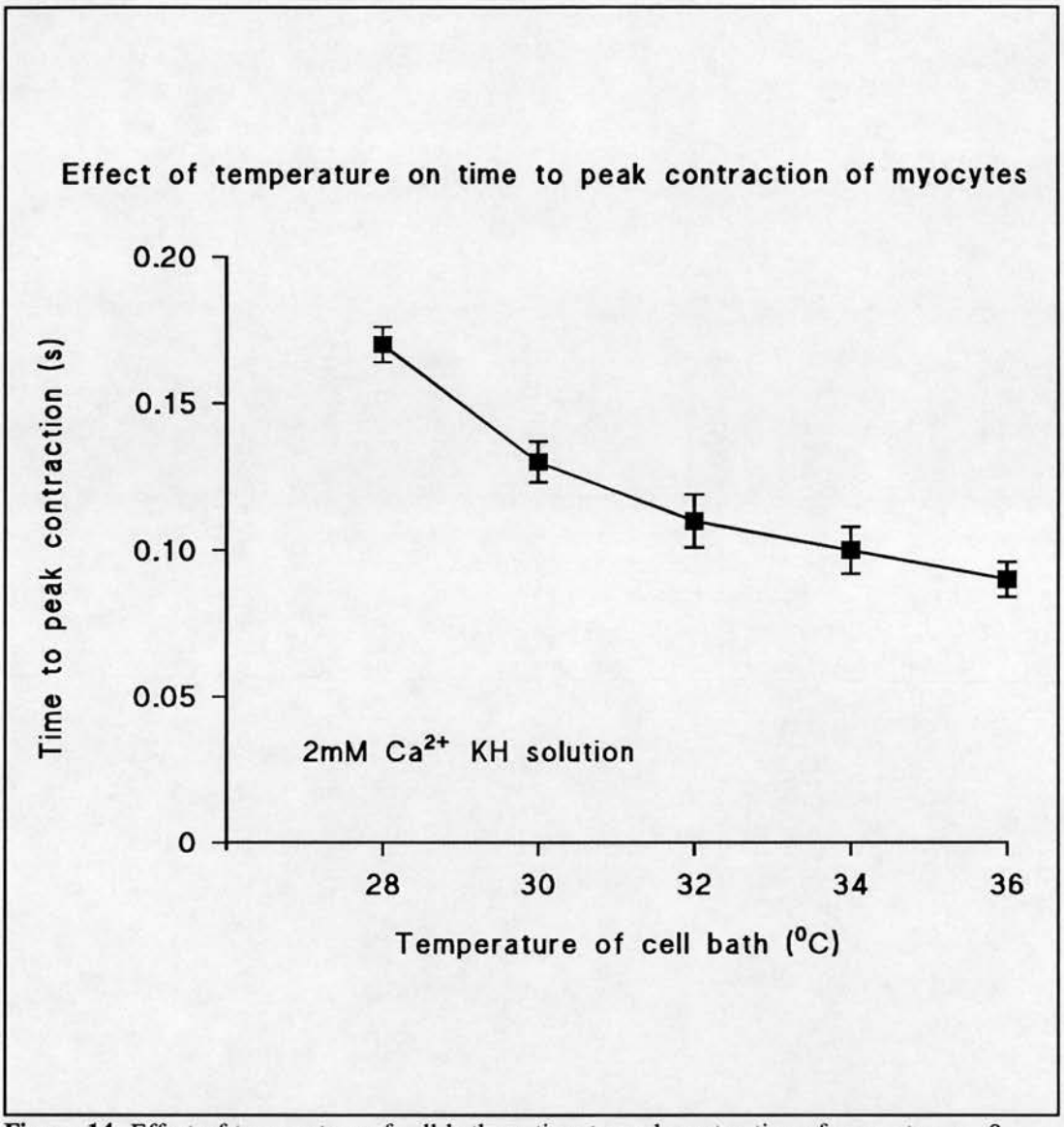


Figure 14 Effect of temperature of cell bath on time to peak contraction of myocytes, n=8.

(0.10±0.02s on endothelium, n=11; 0.12±0.02s on glass, n=9), nor relaxation time (0.30±0.04s on endothelium, n=11; 0.30±0.03s on glass, n=9).

Having established that baseline contraction parameters were not different when myocytes were situated on confluent endothelium subsequent studies could be performed.

It was interesting that myocytes adhered without problem to the endothelial layer. Both cell types express surface glycoproteins, although the majority of these likely appear when the cells are stimulated by cytokines. An alternative explanation for adherence is that myocytes were adhering to glass through gaps between endothelial cells. A further explanation is that the shear force exerted by the buffer

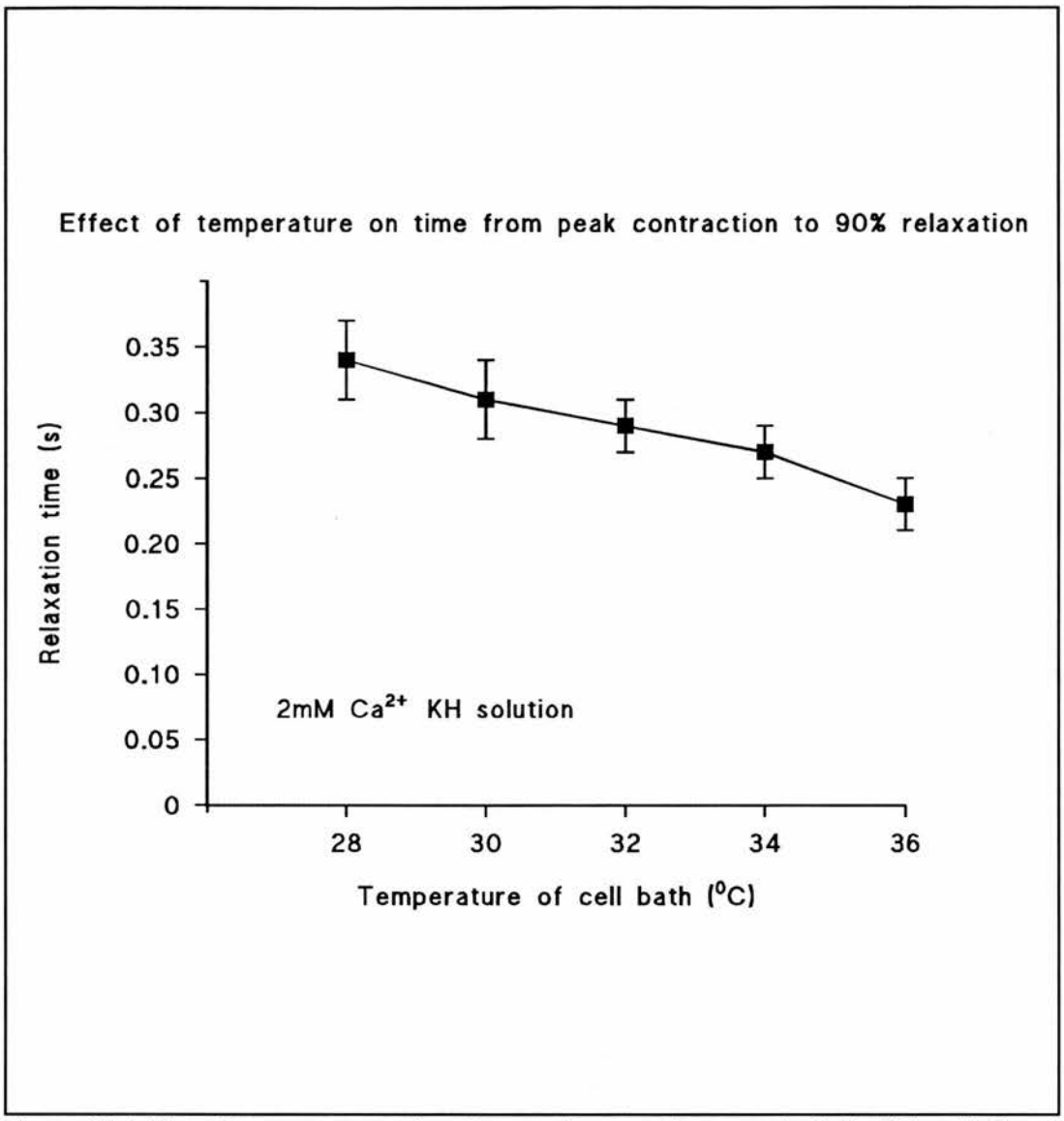


Figure 15 Effect of temperature on time from peak contraction to reach 90% of diastolic length (relaxation time), n=8 cardiac myocytes.

on the surface of the myocyte, transmitted as pressure from the myocyte on the endothelial surface, was less than that necessary to overcome the resistance to movement of the static myocyte sited on a non-friction free surface. Experiments to study this adhesion were not pursued.

CHAPTER 4: EFFECTS OF NITROVASODILATORS AND METHYLENE BLUE ON CARDIAC MYOCYTE CONTRACTILITY

4.1.1. **Introduction**

Endothelium-derived factors, predominantly nitric oxide, modulate blood flow within the vasculature (for review see Moncada (Moncada *et al.*, 1991)). Nitric oxide, synthesised from the amino acid L-arginine by a constitutive nitric oxide synthase enzyme, is released from endothelium and acts rapidly on adjacent vascular smooth muscle to cause relaxation and vasodilatation. This mechanism exists in health to modulate blood flow within tissues. The intracellular pathway involves stimulation of the soluble guanylate cyclase enzyme system within smooth muscle by nitric oxide to increase intracellular levels of cyclic 3',5'-guanosine monophosphate (cGMP), with a subsequent reduction in intracellular calcium. Nitric oxide is capable of relaxing smooth muscle not only within the vascular system, but smooth muscle in airways, gut and urinary tract as well (Moncada *et al.*, 1991).

Evidence is accumulating that the endothelium lining the endocardial surface of the heart can modulate the contraction of myocardial papillary muscle in *in vitro* preparations (Brutsaert *et al.*, 1988; Smith *et al.*, 1991). This specialised endothelial layer produces at least two factors which modify contraction: a substance indistinguishable from endothelium-derived nitric oxide which reduces contractility; and another, as yet unidentified substance which augments contraction.

Within the myocardium, the coronary microcirculation lies in close proximity to cardiac muscle, so that most cardiac myocytes are within 8μm of their nearest capillary (Rose & Goresky, 1984; Randall, 1984). This short diffusing distance means that the contractility of cardiac myocytes making up the bulk of the myocardium might be influenced by vasoactive factors produced by adjacent endothelium, in the same way that endothelial factors regulate smooth muscle contraction in blood vessel walls.

The hypothesis tested is that nitric oxide affects cardiac myocyte contractility.

A secondary aim of the first series of studies was to establish whether cardiac myocytes can metabolise nitrovasodilators. These drugs cause vasodilatation by being metabolised to produce nitric oxide, but different nitrovasodilators are metabolised by different pathways (Furchgott, 1988). Porcine coronary smooth muscle cells metabolise glyceryl trinitrate (GTN) to generate nitric oxide by a plasma membrane enzyme (Chung & Fung, 1990). In contrast, sodium nitroprusside requires only a reduction step requiring NADPH, NADH or a thiol, a reaction which takes place in the microsomes (Fung *et al.*, 1991). Although the effects of nitrovasodilators on cardiovascular haemodynamics are established in both patients and in experimental models, the same haemodynamic changes will obscure any specific action of nitrovasodilators on myocardial contractility, that is, the extent and rate of myocardial shortening. The isolated, functioning cardiac myocyte is thus a suitable model to study contractility in the absence of haemodynamic changes.

4.1.2. **Methods**

Measurement of cardiac myocyte contraction

All experiments were performed on single isolated, contracting, ventricular myocytes. Cells were prepared from isolated, Langendorff perfused guinea-pig hearts by enzymatic digestion, as described previously in chapter 2. A drop of myocyte suspension was placed in a 200 μl perspex chamber on the stage of an inverted microscope on a 22 mm diameter plain glass coverslip and superfused at 2 ml/min with 2 mM calcium Krebs Henseleit (KH) buffer (containing 119.1 mM NaCl, 4.7mM KCl, 0.94 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.5 mM glucose) bubbled with 95%0_2/5%CO_2 at 32$\pm$0.5°C. Temperature was monitored by a thermistor in the cell bath. Cells were allowed to adhere for 4 min without flow and were then stimulated electrically to contract using a bipolar stimulator delivering 30V/ 0.5 ms pulses at 0.5 Hz.

Contractility of individual myocytes was determined as described in detail in chapter 2. Briefly, contraction amplitude and velocity of shortening of electrically-stimulated myocytes were recorded using a videomicroscopy-length detection system and analysed by computer signal-averaging. This system had a time resolution of

20 ms and a spatial resolution of 1 in 256, which allowed typically 10 to 15 sampling points within a single contraction and relaxation. Six consecutive contractions were signal-averaged to produce data under each particular set of conditions. Myocyte contraction amplitude was calculated as a percentage of the resting length, and time to peak contraction, and time from peak contraction to return to 90% of resting length (relaxation time) were recorded.

Criteria were defined to identify healthy, viable, isolated myocytes, listed in chapter 2. (i) only rod-shaped cells without sarcolemmal blebs were examined; (ii) cells which exhibited spontaneous contractions were excluded; and (iii) cells that displayed a variable baseline contraction to electrical stimulation at 2mM calcium were rejected.

Sodium nitroprusside solutions were used over the concentration range 10^{-8}-$3x10^{-5}$M and containers wrapped in foil to exclude light. Concentrations of GTN (Lipha, Middlesex, UK) and ISDN (Schwarz Pharma, Chesham, Bucks., UK) were used over the dose range 10^{-6}-$3x10^{-5}$M. Measurements were made on 6-10 myocytes at each dose of nitrovasodilator. Effects of each nitrovasodilator were compared randomly with control measurements recorded before or after myocyte exposure to nitrovasodilator, at each concentration. Concentrations of nitrovasodilators were chosen randomly, and cumulative dose-response protocols were avoided. Myocytes were exposed to each concentration of nitrovasodilator for 10 min and were not exposed to more than one nitrovasodilator. The time course of the study allowed for measurements made on individual myocytes within 45 minutes of stabilisation. This avoided the reduction in myocyte contraction amplitude that can occur in studies of more than 2 hours' duration.

In further experiments $5x10^{-6}$M methylene blue (David Bull Laboratories, Warwick, UK.) was added as an inhibitor of soluble guanylate cyclase.

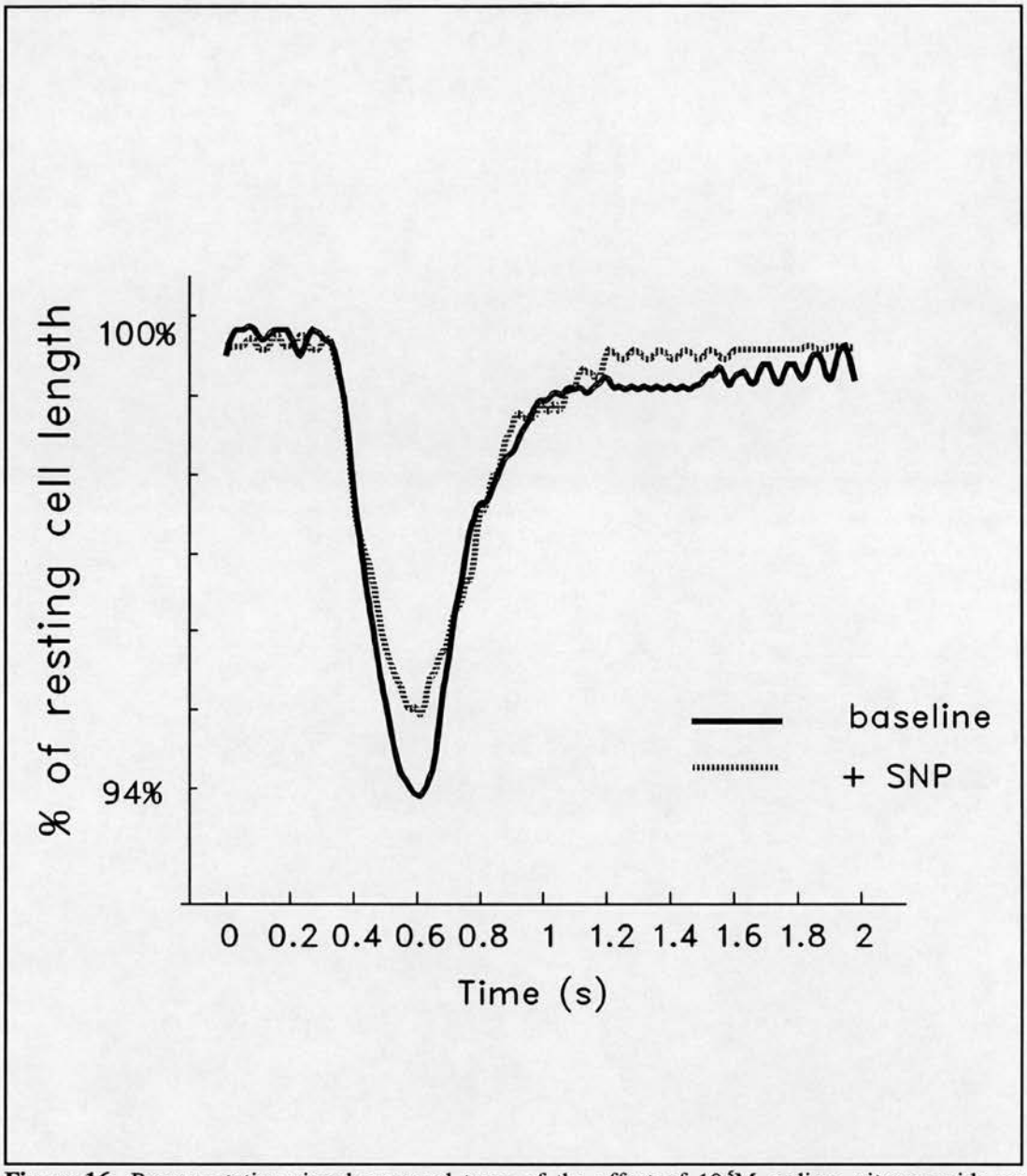


Figure 16 Representative signal-averaged trace of the effect of 10^{-5}M sodium nitroprusside on myocyte contraction.

4.1.3. **Results**

Effects of nitrovasodilators on guinea pig cardiac myocyte contraction.

Figure 16 shows a representative signal-averaged trace (of six contractions) of the effect of 10^{-5}M sodium nitroprusside on contraction of a single myocyte. In this experiment contraction amplitude was reduced from 5.8% to 4.7% of resting length, a reduction in myocyte contraction of 19%, with no effect on either time to

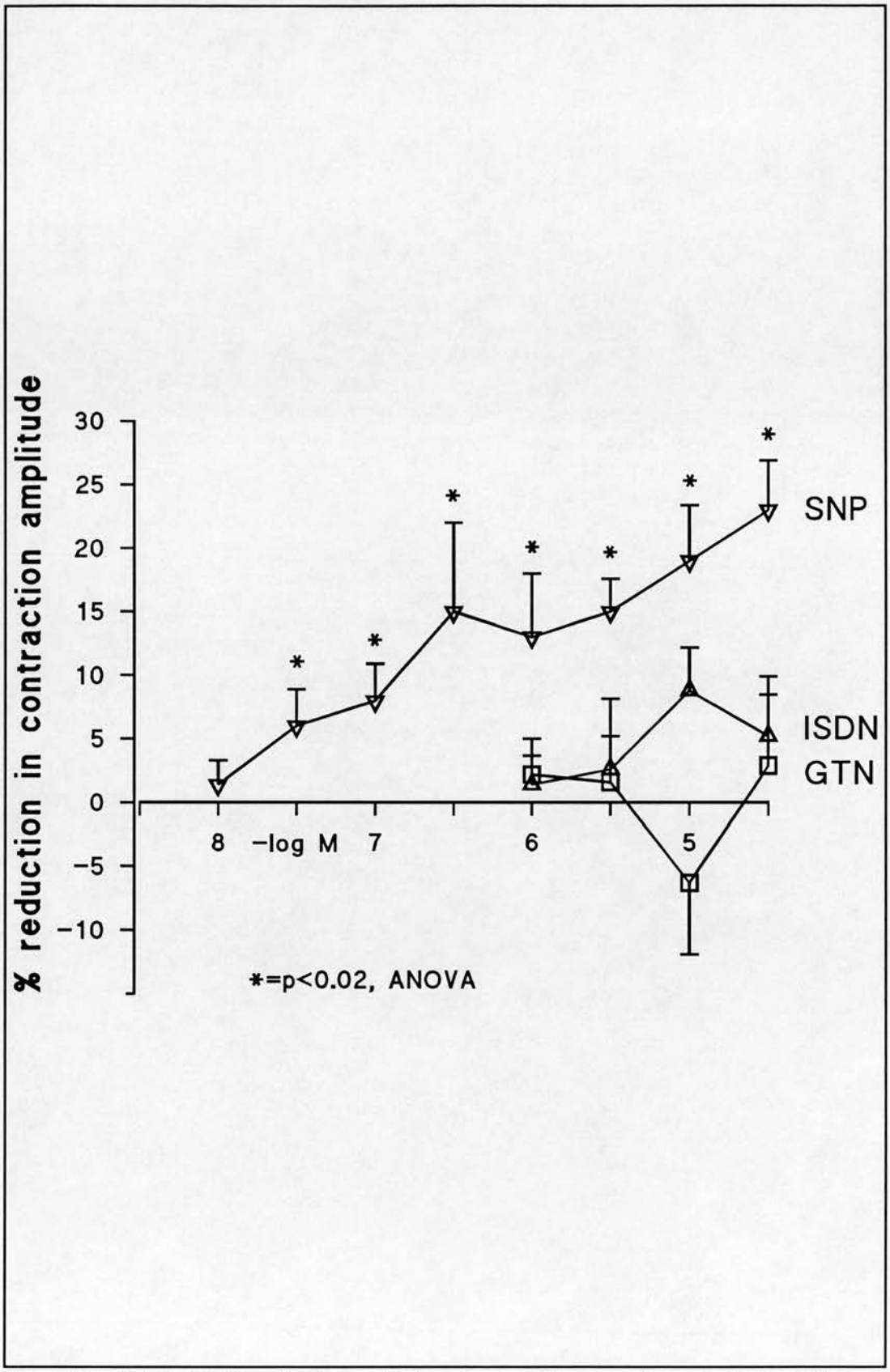


Figure 17 Effect of different nitrovasodilators on myocyte contraction amplitude, differences expressed as percentage reduction of control contractions; mean ± SEM of six-ten experiments.

peak contraction or relaxation time.

The difference between the fractional shortening in the presence of nitrovasodilator was expressed as a percentage of the baseline contraction (Figure 17). GTN and ISDN over the dose range 10^{-6}-$3x10^{-5}$M had no effect on contraction amplitude of contracting myocytes.

Sodium nitroprusside reduced contraction amplitude: the reduction was significant ($p<0.02$, ANOVA) at concentrations of 3 x 10^{-8}M or above. Regression analysis showed that the effect of sodium nitroprusside was concentration dependent ($P<0.001$), although it was not possible to determine whether the curve was monophasic or biphasic.

In further studies $5x10^{-6}$M methylene blue was added to all solutions and reversed the reduction in myocyte shortening caused by sodium nitroprusside (Figure 18). In control studies, methylene blue itself had no effect on myocyte contractility over the same time course as these experiments. However, superfusion of methylene blue for more prolonged periods (>30-60 min) caused cells to become hypercontractile and subsequently fibrillate in ten of twelve cardiac myocytes studied.

Compared to control, none of the nitrovasodilators had a significant effect on time to peak contraction (baseline, 0.14 ± 0.02 s; nitrovasodilator, 0.14 ± 0.02 s, mean$\pm$SD; pooled data from all nitrovasodilator experiments, $n=12$ groups of paired studies). Similarly, relaxation times were not affected by nitrovasodilators (baseline 0.27 ± 0.05 s; nitrovasodilator 0.28 ± 0.04 s, mean$\pm$SD, pooled data from the same experiments. This data is shown in Table 4 below, and graphically in Figures 19-24.

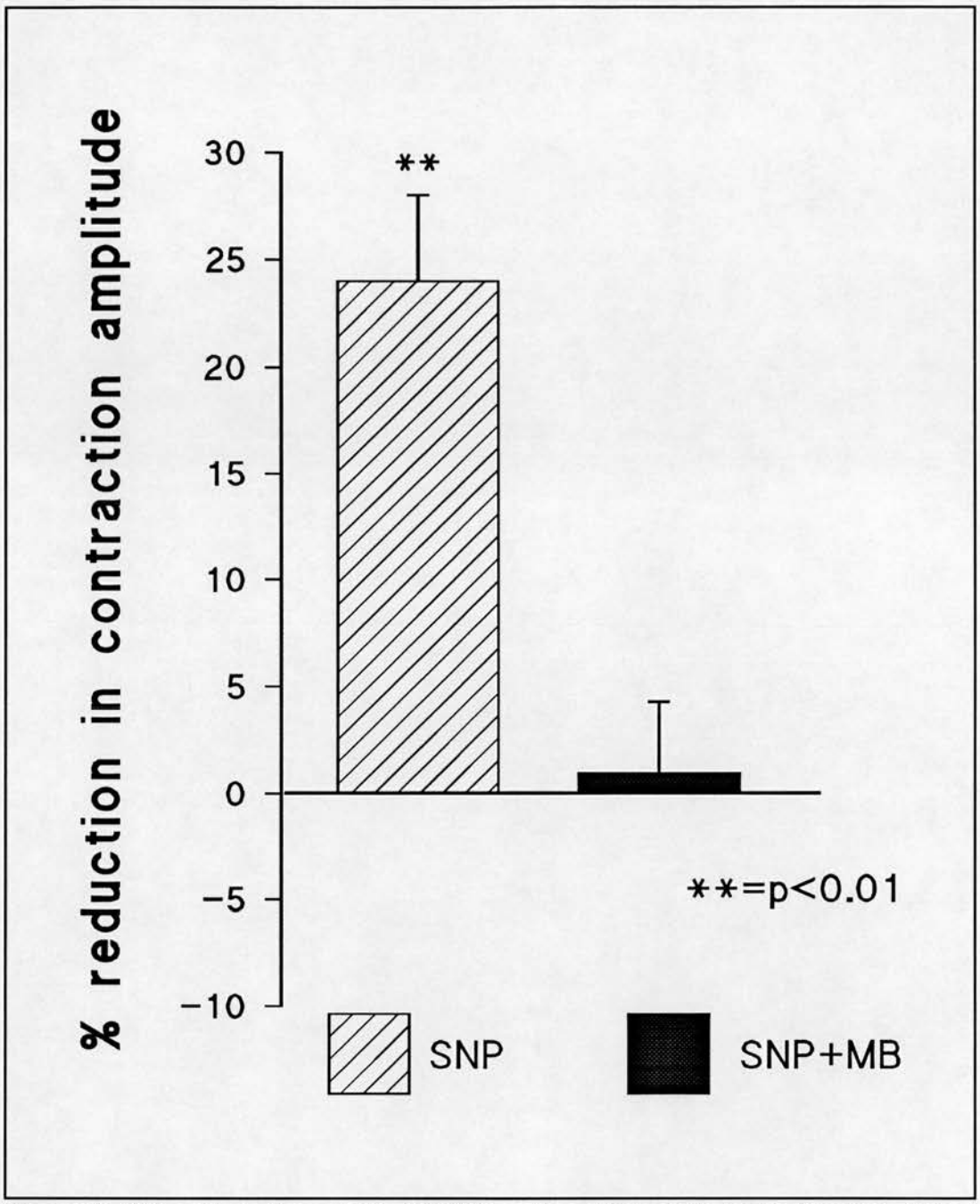


Figure 18 SNP, sodium nitroprusside; SNP/MB, sodium nitroprusside + methylene blue. Values are mean ± SEM of six experiments. **=$p < 0.01$, both SNP versus control and SNP versus SNP + MB.

Table 4

Effects of Glyceryl Trinitrate, Isosorbide Dinitrate and Sodium Nitroprusside on Time to Peak Contraction and on Relaxation Time of Isolated, Functioning Guinea-pig Cardiac Myocytes.

	Time to peak contraction (s)		Relaxation time (s)	
	control	nitrovasodilator	control	nitrovasodilator
10^{-6}M GTN	0.13±0.02	0.13±0.02	0.24±0.02	0.23±0.02
$3x10^{-6}$M GTN	0.12±0.02	0.13±0.02	0.24±0.02	0.24±0.02
10^{-5}M GTN	0.12±0.02	0.13±0.02	0.21±0.02	0.24±0.02
$3x10^{-5}$M GTN	0.13±0.02	0.13±0.02	0.22±0.02	0.23±0.02
10^{-6}M ISDN	0.15±0.02	0.15±0.02	0.32±0.03	0.32±0.03
$3x10^{-6}$M ISDN	0.14±0.02	0.14±0.02	0.32±0.05	0.33±0.04
10^{-5}M ISDN	0.15±0.02	0.15±0.02	0.26±0.03	0.25±0.03
$3x10^{-5}$M ISDN	0.14±0.02	0.15±0.02	0.23±0.02	0.24±0.02
10^{-6}M SNP	0.15±0.02	0.15±0.02	0.35±0.04	0.34±0.04
$3x10^{-6}$M SNP	0.15±0.02	0.16±0.02	0.28±0.03	0.31±0.04
10^{-5}M SNP	0.12±0.02	0.11±0.02	0.30±0.03	0.30±0.03
$3x10^{-5}$M SNP	0.12±0.02	0.12±0.02	0.31±0.10	0.31±0.03

Values are mean±SEM of six-nine paired experiments at each concentration of nitrovasodilator (except $3x10^{-5}$M SNP, n=4 experiments). GTN, glyceryl trinitrate; ISDN, isosorbide dinitrate; SNP, sodium nitroprusside.

Effect of sodium nitroprusside on contractility of human cardiac ventricular myocytes.

The opportunity was taken to confirm the effect of the nitric oxide donor, sodium nitroprusside, on human cardiac ventricular myocytes. Three cells from two patients with dilated cardiomyopathy were studied. Contractility was reduced in a manner similar to guinea pig myocytes. The magnitude of reduction was similar to that seen with guinea pig myocytes, as shown in Figure 25.

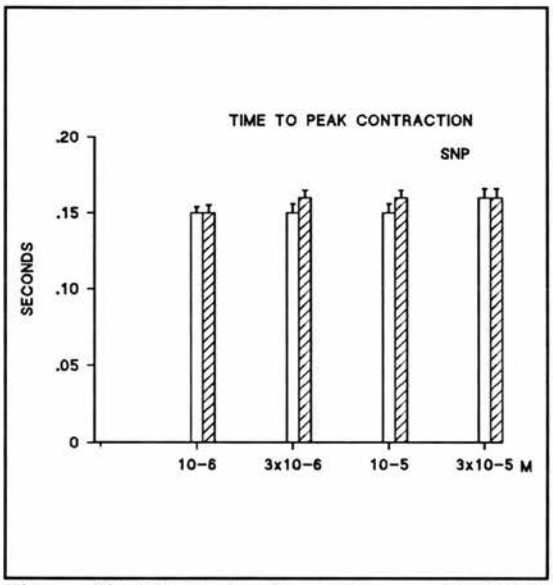


Figure 19 Effect of sodium nitroprusside (SNP) on time to peak contraction, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of SNP.

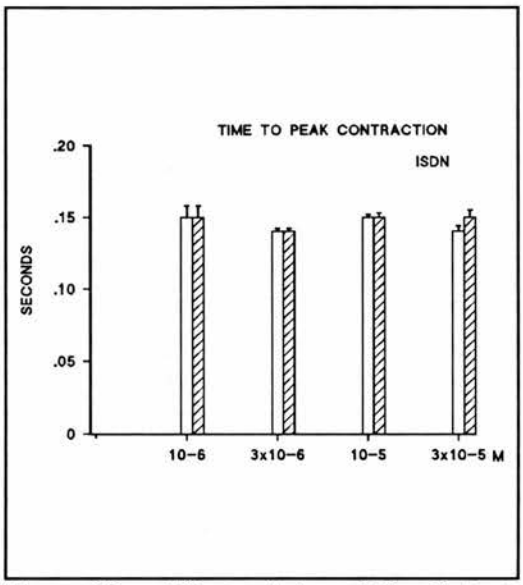


Figure 20 Effect of isosorbide dinitrate (ISDN) on time to peak contraction, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of ISDN.

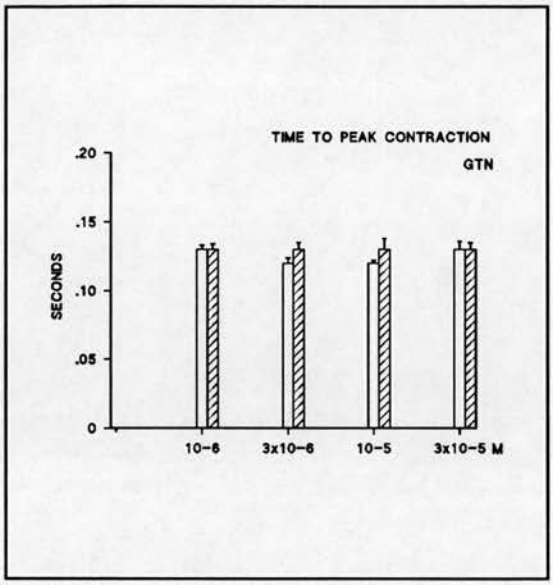


Figure 21 Effect of glyceryl trinitrate (GTN) on time to peak contraction, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of GTN.

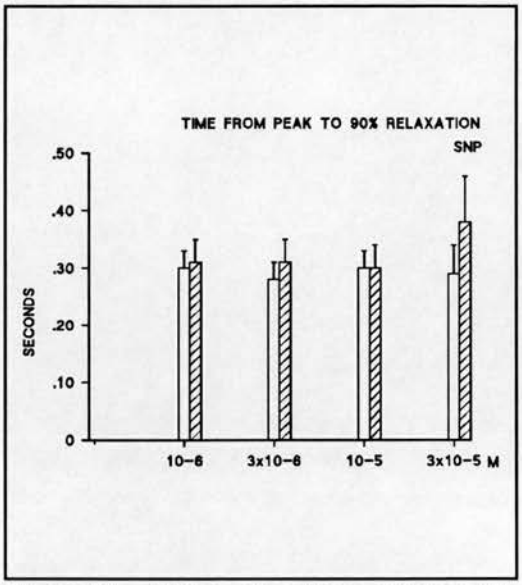


Figure 22 Effect of sodium nitroprusside (SNP) on time from peak contraction to 90% relaxation, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of SNP.

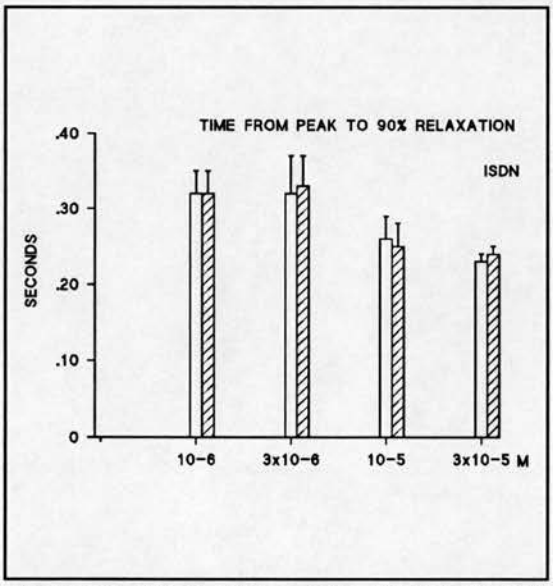


Figure 23 Effect of isosorbide dinitrate (ISDN) on time from peak contraction to 90% relaxation, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of ISDN.

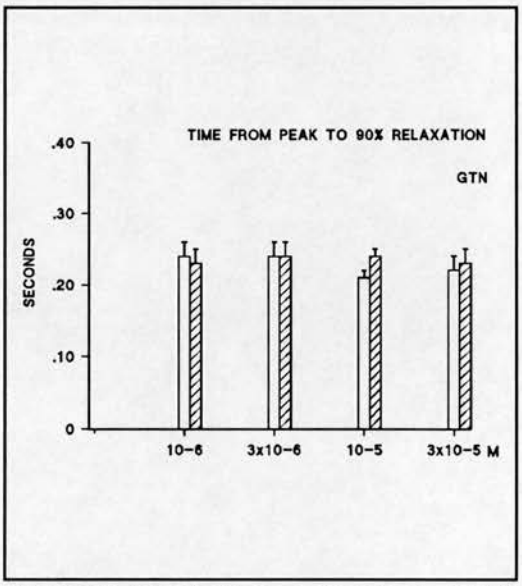


Figure 24 Effect of glyceryl trinitrate (GTN) on time from peak contraction to 90% relaxation, n=6-9 myocytes at each concentration. Open bars=control; hatched bars= after addition of GTN.

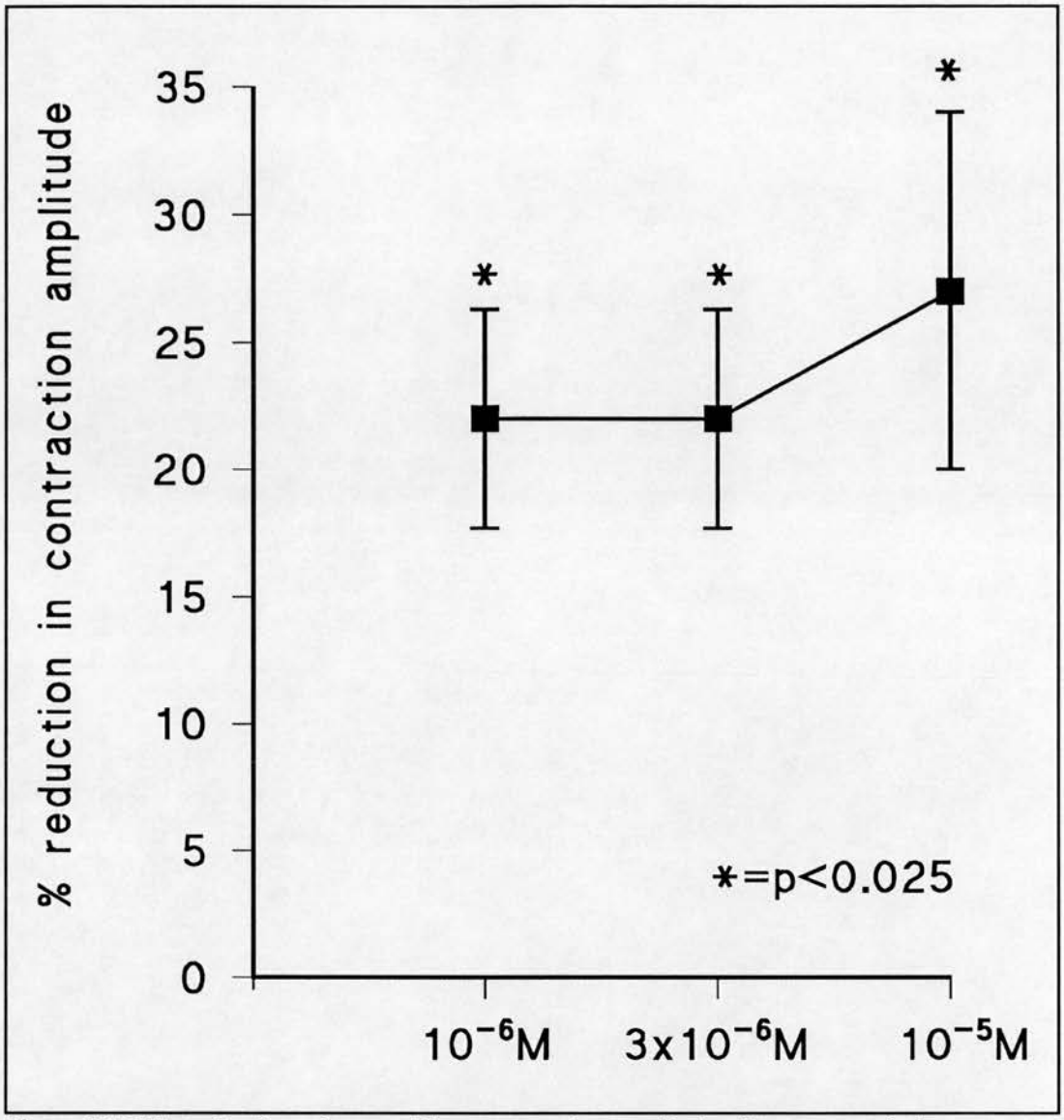


Figure 25 Effect of sodium nitroprusside on contraction amplitude of human ventricular myocytes, differences expressed as percentage reduction of baseline contraction; mean ± SEM of three studies.

4.1.4. **Discussion**

Sodium nitroprusside reduced the contraction amplitude of isolated myocytes in the present study. This effect was reversed by the guanylate cyclase inhibitor, methylene blue, consistent with it being mediated by generation of nitric oxide from sodium nitroprusside with subsequent elevation of intracellular cGMP. The effect of sodium nitroprusside was reversible and repeatable, suggesting that the generation of cyanide was not a cause of the reduced contraction amplitude.

The opportunity to confirm these findings in human ventricular myocytes arose. Sodium nitroprusside reduced contractility in an identical fashion to that seen in guinea pig cells.

The effect of sodium nitroprusside in papillary muscle (Smith *et al.*, 1991) is of similar magnitude to the reduction in contraction seen in the present study. In papillary muscle experiments, small changes in time-dependent variables can be demonstrated. These changes are within 20 ms (Smith *et al.*, 1991), and while our studies detected no effects on either times to peak contraction or relaxation times, this may be because the time resolution of our videomicroscopy edge-detection device is itself of the order of 20 ms.

In the present study methylene blue reversed the effect of sodium nitroprusside over a short time course. Toxicity was not observed during exposure of myocytes to methylene blue for periods less than 10 min. However, in the studies on myocytes isolated from guinea pigs with endotoxaemia exposure of these cells to methylene blue over 10-15 min resulted in inotropic, then toxic effects (see Chapter 7). In another recent study methylene blue (10 μM) administered to both neonatal and adult rat cardiac myocytes had no effect on basal contractility, but enhanced the inotropic effects of isoprenaline (Balligand *et al.*, 1993). Toxic effects were not described, although the time course of exposure of cells to methylene blue was not noted in their paper. Their interpretation of the effects of methylene blue was that a constitutive nitric oxide synthase was active during ß-adrenergic stimulation and may mediate in part cholinergic regulatory mechanisms.

It is of interest that sodium nitroprusside, but not the organic, and more commonly used nitrates, ISDN and GTN, was metabolised to generate nitric oxide within cardiac myocytes in the present study. Hence cardiac myocytes may not contain the enzymes necessary to produce nitric oxide from the latter two drugs. This difference in metabolism may be masked *in vivo* by the widespread haemodynamic changes following nitrovasodilator administration. In patients with unstable angina, sodium nitroprusside showed no difference with respect to haemodynamic changes when compared to GTN (Breisblatt *et al.*, 1988); it may be that vascular smooth muscle is more sensitive than cardiac muscle to the actions of sodium nitroprusside, and thus vasodilator effects are seen before changes in myocardial contractility

develop. In patients with angina pectoris, the therapeutic concentration range in plasma of intravenous isosorbide dinitrate is approximately $4x10^{-9}M$ - $10^{-8}M$ (manufacturer's information, Schwartz Pharma, Chesham, Bucks). Sodium nitroprusside is used to control blood pressure in anaesthesia over the plasma concentration range $8x10^{-8}M$ - $9x10^{-7}M$ (Vesey *et al.*, 1990). Thus vascular smooth muscle *in vivo* is about 10-100 fold more sensitive than cardiac tissue *in vitro* to the effects of nitrovasodilators.

GTN and ISDN generate nitric oxide in the epicardial coronary circulation. Yet no effect on myocardial contractility occurs when nitrates are injected down coronaries. An interesting question is: why is myocardial contractile function *not* altered visibly by direct intracoronary injection of nitrate, a procedure frequently performed at coronary angiography? Presumably, while some GTN or ISDN is evidently metabolised to release nitric oxide by the endothelium and vascular smooth muscle, the amount generated locally is limited in its effect to the same vascular lining cells, without reaching the ventricular myocytes. Alternatively, the same explanation for the study just mentioned (Breisblatt *et al.*, 1988) may be involved. Systemic effects may be apparent before cardiac effects are achieved. In animals, the coronary circulation has been shown to be less sensitive to the effects of GTN than the peripheral vasculature (Sellke *et al.*, 1990).

The mechanism by which elevation of cGMP may cause a reduction in myocyte contraction is not well understood. Possible pathways are discussed further in chapters 5 and 9.

Nitrovasodilators thus differ in their ability to affect cardiac myocyte contractility. The results of this study led me to proceed to develop a coculture of endothelium with cardiac myocytes, and to further studies described in the next five chapters.

CHAPTER 5: EFFECT OF NITRIC OXIDE AND 8-BROMO-CYCLIC GMP ON CARDIAC MYOCYTE CONTRACTILITY

5.1.1. **Introduction**

Nitric oxide synthase is constitutively expressed in many cell types. These include some neural tissues; circulating neutrophils, mast cells and platelets; pancreatic islet ß cells; and renal macula densa cells, as well as endothelium (Moncada *et al.*, 1991; Nathan, 1992). Production of nitric oxide appears to be a fundamental and important mechanism of intercellular signalling in these cell types, and nitric oxide modulates or mediates cellular function in a variety of tissues.

The experiments in chapter 4 suggested that nitric oxide derived from sodium nitroprusside attenuated cardiac myocyte contraction. The hypothesis again tested in the present study is that nitric oxide affects cardiac myocyte contractility. Nitric oxide directly in aqueous solution superfused over contracting cardiac myocytes is the ultimate straightforward test of this hypothesis.

Nitric oxide acts in the vasculature by elevating intracellular levels of cGMP. In the previous study, methylene blue, a nonspecific inhibitor of guanylate cyclase, reversed the effects of sodium nitroprusside, suggesting the action of the nitrovasodilator was mediated by elevation of cGMP levels within the myocytes. In the present study the effects of the stable analogue, 8-bromo-cyclic GMP were therefore studied. 8-bromo-cyclic GMP is a lipid soluble, stable analogue of cGMP. It can cross cell membranes and is used experimentally to mimic elevation of cGMP.

5.1.2. **Methods**

Measurement of cardiac myocyte contraction

All experiments were performed on single isolated, contracting, ventricular myocytes. Cells were prepared from isolated, Langendorff perfused guinea-pig hearts by enzymatic digestion, as described previously, in chapter 2.

Contractility of individual myocytes was determined as described in detail in chapter 2. Criteria were defined to identify healthy, viable, isolated myocytes: (i)

only rod-shaped cells without sarcolemmal blebs were examined; (ii) cells which exhibited spontaneous contractions were excluded; and (iii) cells that displayed a variable baseline contraction to electrical stimulation at 2mM calcium were rejected.

Preparation of aqueous nitric oxide

Experiments were performed to examine the effect of nitric oxide dissolved in physiological solution on cardiac myocyte contraction. 100 μl or 1000 μl aliquots of nitric oxide gas (99% pure; Merck, Poole, Dorset, UK) were injected using a Hamilton gas syringe (Reno, Nevada) into 300 ml HEPES buffered 2mM Ca^{2+} Tyrodes solution (containing 150 Mm NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 5.0 mM Na-HEPES, 10.0 mM glucose; pH 7.40) which had been bubbled vigorously with nitrogen gas for 4 h to remove oxygen. This yielded stock solutions of approximately 10^{-6}-10^{-5}M dissolved nitric oxide, respectively, and the stock solutions were kept anoxic. Aliquots were withdrawn and superfused into the cell bath for 7 min. The concentration of the 10^{-6}M nitric oxide solutions was confirmed using a Sievers Nitric Oxide Chemiluminescence Analyzer (Denver, Colorado) to be within the range 1-3x10^{-6}M on multiple samplings of different 100 μl solutions. The 1000 μl solutions were assumed to contain tenfold more nitric oxide. Control experiments were performed using the same Tyrodes solution, bubbled with 100% nitrogen.

5.1.3. **Results**

Figure 26 shows a representative trace of six signal-averaged contractions of the effect of 10^{-5}M nitric oxide solution on contraction amplitude of a single cardiac myocyte. Figure 27 shows the effect of direct superfusion with 10^{-6}M and 10^{-5}M nitric oxide solution on myocyte contraction amplitude, compared to control, of five experiments. Nitrogen gassed control solution had no effect on myocyte contraction (n=13 myocytes) so the effect of the nitric oxide solution was not due to hypoxia. Administration of 10^{-5}M nitric oxide did not have a greater effect than 10^{-6}M nitric oxide. The reduction in contractility seen with the nitric oxide solutions was similar to that caused by 3x10^{-5}M sodium nitroprusside.

Time dependent variables were measured (Figures 28 and 30). There was

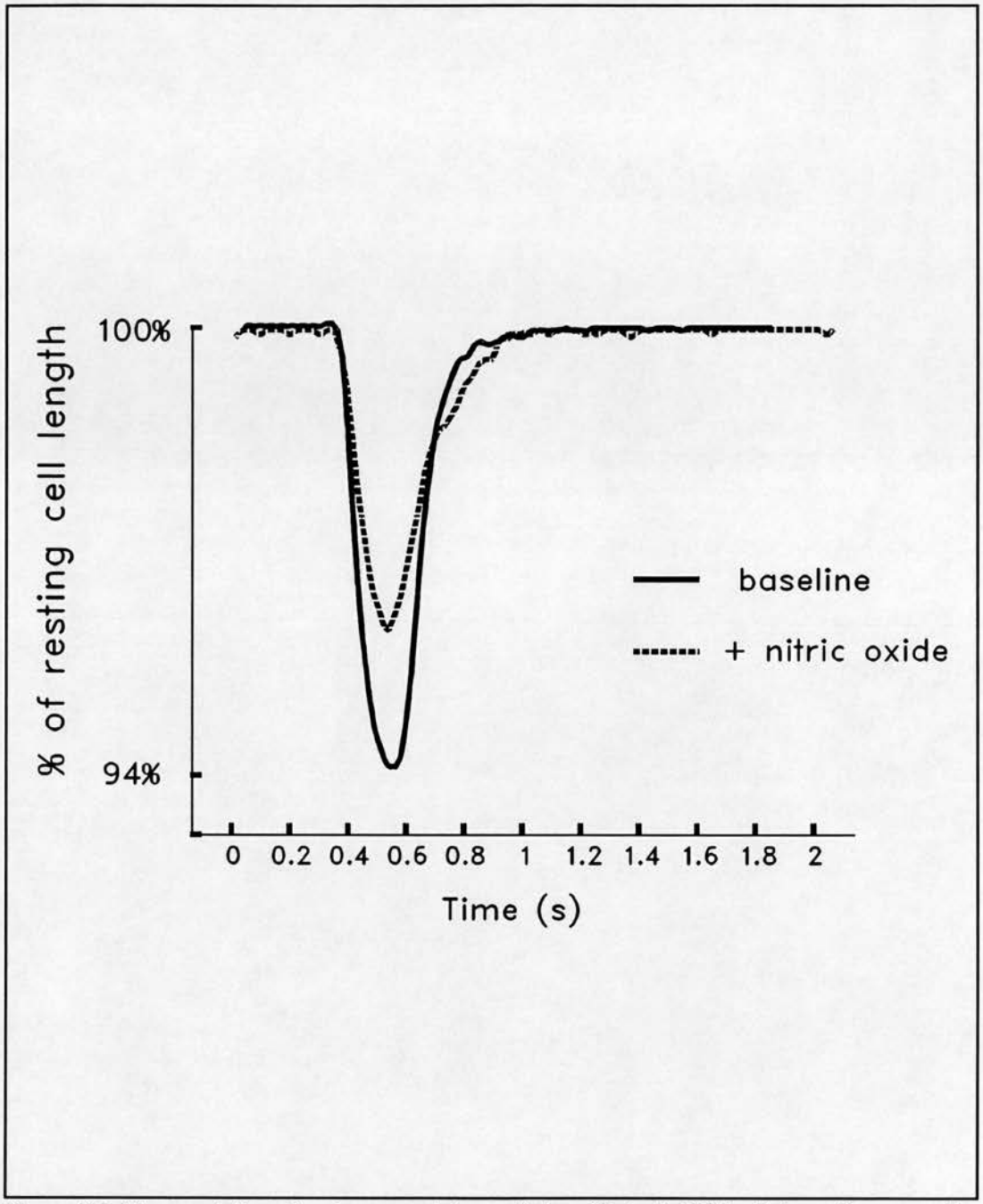


Figure 26 Representative signal averaged trace of the effect of 10^{-5}M nitric oxide on cardiac myocyte contraction.

slight reduction in relaxation time at one of the doses of nitric oxide solution. This effect on relaxation time was not seen with sodium nitroprusside, nor with stimulated endothelium. Its significance is uncertain. This reduction was not seen with 8-bromo-cyclic GMP either, although this drug reduced time to peak contraction slightly at 10^{-5}M, but not at other doses (Figure 30).

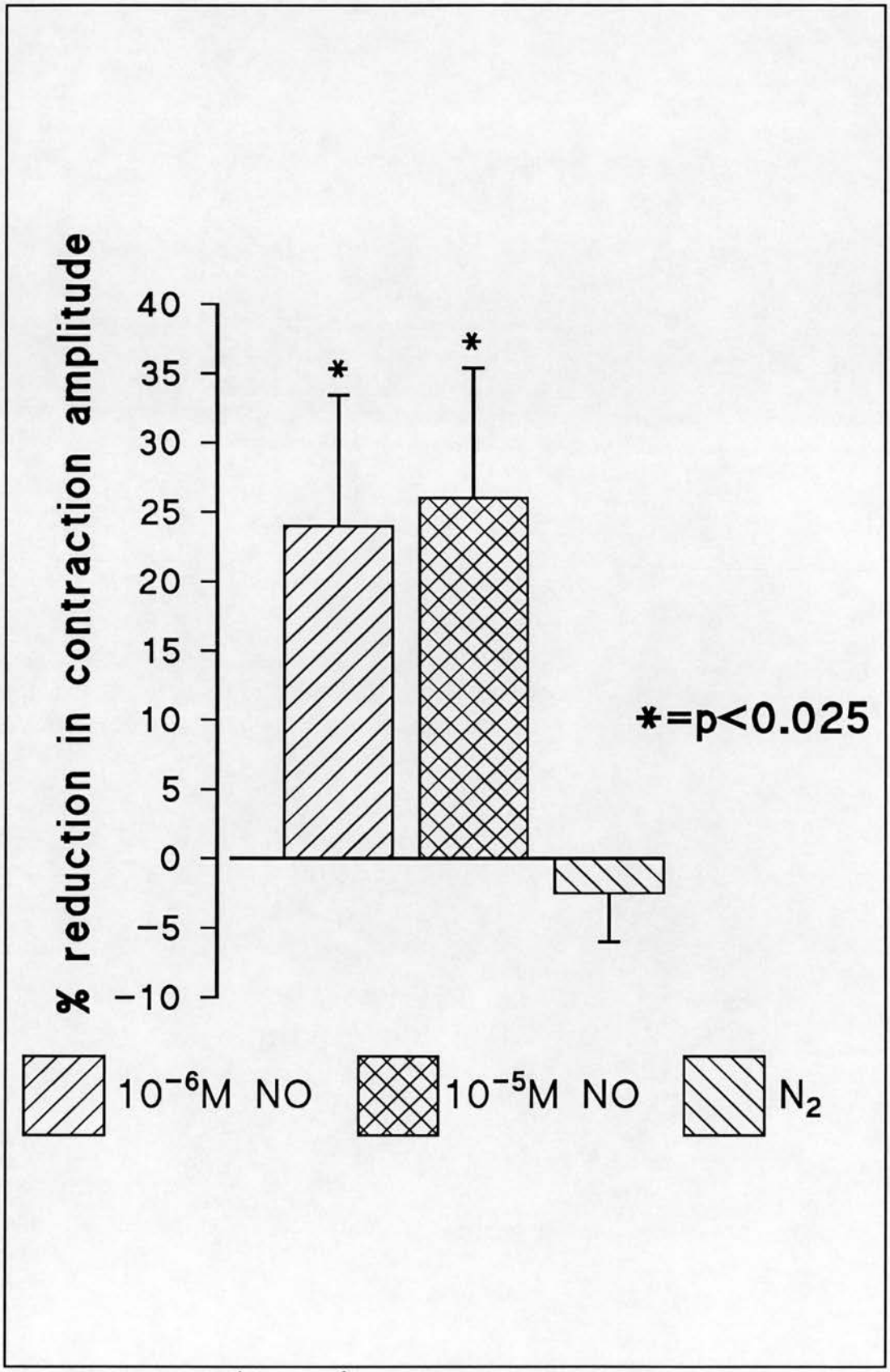


Figure 27 Effect of 10^{-6}M and 10^{-5}M NO solution, and N_2 solution on myocyte contraction. Mean ± SEM of five (NO) and 13 (N_2) experiments.

The stable cGMP analogue, 8-bromo-cyclic GMP, caused a concentration-related reduction in myocyte contraction amplitude, similar in magnitude to the effect of sodium nitroprusside shown in chapter 4 (Figure 17). Effects of 8-bromo-cyclic GMP on time to peak contraction and relaxation time are shown in Figure 29. I tested 8-bromo-cyclic GMP on two human ventricular myocytes. Administration of 10^{-7}M and 10^{-6}M solutions decreased contraction amplitude by approximately 25% in each case.

Effect of 10^{-6}M and 10^{-5}M nitric oxide on time to peak contraction and 90% relaxation time of cardiac ventricular myocytes

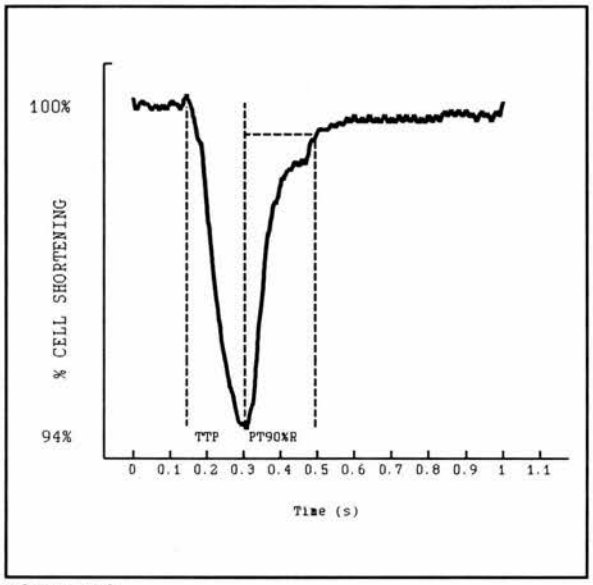


Figure 28

	Time to peak contraction (s)	Relaxation time (s)
baseline	0.12±0.02s	0.49±0.07s
10^{-6}M NO	0.11±0.02s; p=NS	0.37±0.06s; p<0.01
10^{-5}M NO	0.12±0.02s; p=NS	0.45±0.03; p=NS

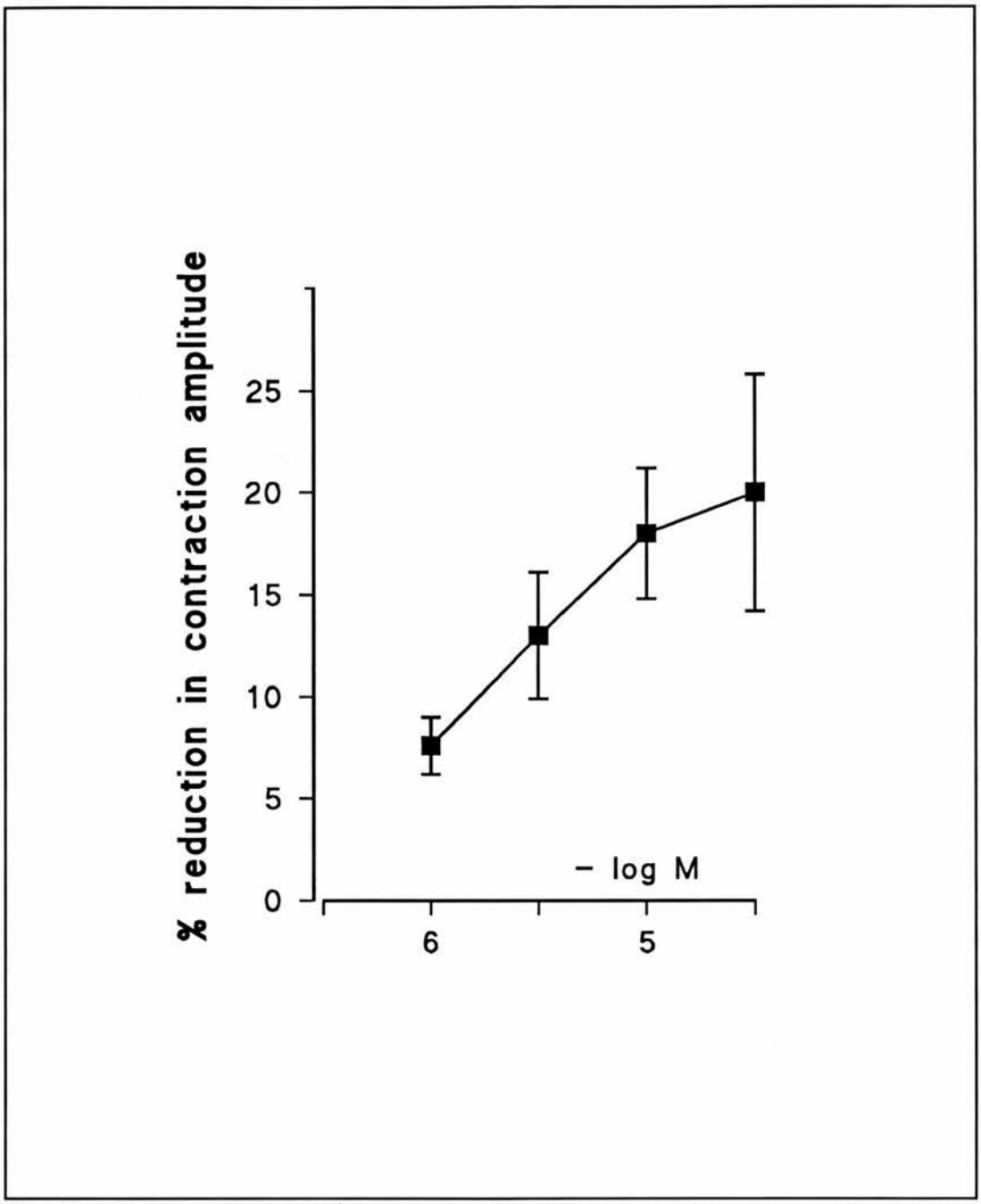


Figure 29 Effect of 10^{-6}M-3x10^{-5}M 8-bromo-cyclic GMP on myocyte contraction amplitude, as a percentage of baseline contraction. Mean $\pm$ SEM of five-seven experiments; $p < 0.025$ at each dose.

Effect of 8-bromo-cyclic GMP on time to peak contraction and 90% relaxation time of cardiac ventricular myocytes

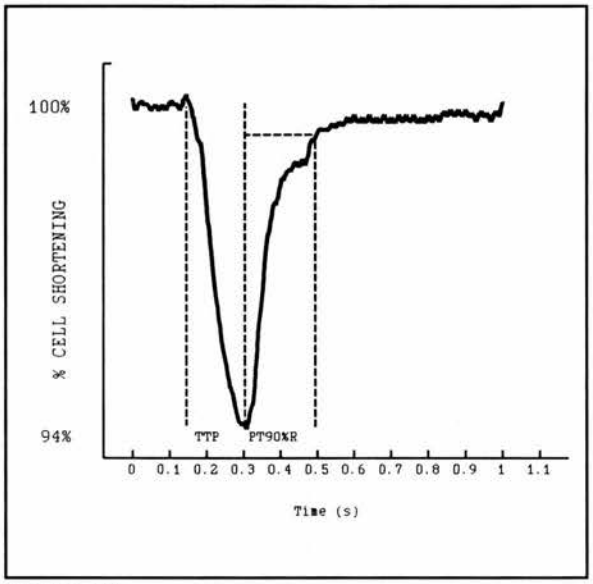


Figure 30

	Time to peak contraction (s)	Relaxation time (s)
baseline	0.13±0.01s	0.32±0.03s
10^{-6}M 8-bromo-cGMP	0.12±0.02s; p=NS	0.34±0.02s; p=NS
10^{-5}M 8-bromo-cGMP	0.11±0.02s; p<0.05	0.27±0.05s; p=NS

5.1.4. **Discussion**

The experiments show that nitric oxide in aqueous solution directly causes a reduction in cardiac myocyte contraction amplitude. This was not caused by anoxia because the control solution containing only N_2 was without effect. The open design of the cell bath allowed adequate oxygenation from the atmosphere. Although the amount of nitric oxide converted to nitrite on exposure to air within the cell bath is unknown, because the nitric oxide solution was protected from exposure to air until it reached the cell under study, sufficient nitric oxide from a solution of approximately 10^{-6}M could reach the myocytes unchanged with an effect similar to $3x10^{-5}$M sodium nitroprusside. It appears that 10^{-6}M nitric oxide in aqueous solution had a maximum effect, since 10^{-5}M solution did not significantly reduce further the contraction amplitude.

Nitric oxide acts within cells by stimulating soluble guanylate cyclase to increase levels of cGMP, and in vascular smooth muscle this can be inhibited predictably by methylene blue. Haemoglobin is used in vascular preparations to bind free nitric oxide, and this ability was recognised early in the development of nitric oxide biology. Proteins froth due to bubble formation at the electrodes in the cell bath, so haemoglobin was not used.

Exposure of myocytes to the stable analogue of cGMP, 8-bromo-cyclic GMP, had an effect similar to nitric oxide either administered in aqueous solution or derived from sodium nitroprusside. A relatively high concentration of 8-bromo-cyclic GMP was needed, consistent with the natural amplification of a biological signal, whereby receptor-mediated events, for example stimulation by nitric oxide, are amplified by the second messenger systems. Alteratively, this may reflect the difficulty of entry of 8-bromo-cyclic GMP into myocytes. The same analogue has been tested in endothelium-intact papillary muscles (Shah & Henderson, 1992). A negative inotropic effect similar to that seen in the present study was observed. 10^{-4}M 8-bromo-cyclic GMP was required in their study to produce the negative inotropic effect on papillary muscle contraction.

The mechanism by which elevation of cGMP may cause a reduction in cardiac myocyte contraction is not well understood. In frog cardiac myocytes, cGMP inhibits the L-type inward calcium channel current (I_{Ca}) by stimulation of cyclic 3'5'-adenosine monophosphate (cAMP) phosphodiesterase, but in rat ventricular myocytes cGMP predominantly inhibits this calcium current by a mechanism involving cGMP-dependent protein kinase, independent of any effect on cAMP levels (Walter, 1989; Lohmann *et al.*, 1991). Furthermore, cGMP has both stimulatory and inhibitory actions on different phosphodiesterases in rat cardiac myocytes and under certain experimental conditions has a stimulatory effect on I_{Ca} (Lohmann *et al.*, 1991). I studied the effect of nitric oxide on calcium transients recorded during contraction, and these results, together with further discussion of the mechanism of cGMP action within myocytes, are presented in Chapter 9.

In the present study a maximal effect occurred with the lowest dose of nitric oxide solution, *i.e.* 10^{-6}M. When I designed the study the available data from my nitrovasodilator experiments suggested that a reliable reduction in myocyte contractility was obtained with administration of 10^{-6}-10^{-5}M solutions. This accounts for the concentrations of nitric oxide chosen for the present study. Stoichiometrically, one nitroprusside molecule releases one nitric oxide molecule. Generation of nitric oxide from nitroprusside requires a reduction step which takes place in the microsomes. Nitric oxide was at least 10 fold more potent than nitroprusside in my experiments, and this may reflect incomplete metabolism of nitroprusside by myocytes. In both the sodium nitroprusside and nitric oxide studies the reduction in contractility was reversible, which makes this effect unlikely to be due to toxicity.

I would have liked to have examined the effects of lower concentrations of nitric oxide to establish a dose-response curve. Threshold changes in contractility are smaller and greater numbers would be required for accuracy. Only one dose of nitric oxide solution could be studied each day, because preparation of solutions was lengthy and only one concentration could be performed each day. Moreover, nitric oxide is expensive. A clear effect was demonstrated at 10^{-6}M, so studies at lower concentrations were not pursued.

Superfusion with anoxic 2 mM Tyrode's solution had no effect compared to baseline. While this result was an important control, supportive of the data from

nitric oxide solution experiments, it meant that to study hypoxia-reperfusion at cellular level, simply using hypoxic buffer would have no effect. The open nature of the cell bath allowed adequate oxygenation from room air. The ways I addressed hypoxia to study myocardial stunning at a cellular level are discussed in chapter 8.

CHAPTER 6: CARDIAC MYOCYTE CONTRACTILITY IN PRIMARY COCULTURE WITH ENDOTHELIUM

6.1.1. **Introduction**

Endothelium-derived factors modulate blood flow within the vasculature. Nitric oxide, synthesised from the amino acid L-arginine by a constitutive nitric oxide synthase enzyme, is released from endothelium and acts rapidly on adjacent vascular smooth muscle to cause relaxation and vasodilatation. This mechanism modulates blood flow within tissues. The intracellular pathway involves stimulation of the soluble guanylate cyclase enzyme system within smooth muscle by nitric oxide to increase intracellular levels of cyclic 3',5'-guanosine monophosphate (cGMP), with a subsequent reduction in intracellular calcium.

Within the myocardium, the coronary microcirculation lies in close proximity to cardiac muscle, so that most cardiac myocytes are within 8 μm of their nearest capillary, as illustrated in chapter 1.4.2.. This short diffusing distance means that the contractility of cardiac myocytes making up the bulk of the myocardium might be influenced by vasoactive factors produced by adjacent endothelium, in the same way that endothelial factors regulate smooth muscle contraction in blood vessel walls.

The hypothesis tested is that nitric oxide affects cardiac myocyte contractility. For this series of experiments I developed a coculture of myocytes together with endothelial cells to examine if there was a direct effect of endothelial factors on myocyte contraction.

Since endothelial cells can release prostacyclin and other prostaglandins I performed some experiments in the presence of indomethacin. Prostaglandins have inotropic effects on myocardium, but only at high pharmacological concentrations (Alloatti *et al.*, 1991; Otani & Das, 1988). The effect of Iloprost, a stable analogue of prostacyclin, was examined in control myocytes without endothelium.

6.1.2. **Methods**

Endothelium-cardiac myocyte coculture model

Bovine aortic endothelial cells were isolated and passaged by non-enzymatic methods, as described in chapter 2. The cells had normal cobblestone morphology, Factor VIII immunoreactivity and high levels of angiotensin-converting enzyme activity. They were used between passages 10-18 and grown in Dulbecco's modified Eagle's medium with 10% fetal-calf serum (Gibco, Paisley, Scotland), penicillin 200,000 IU/l, streptomycin 200 mg/l and glutamine 480 mg/l. Cells were passage on to sterile glass coverslips contained in multiwell plates and used 2-3 days later when confluent.

In the endothelium-myocyte coculture experiments 10^{-7}M bradykinin was used to stimulate release of endothelial-derived factors. The ability of the endothelium to generate nitric oxide when stimulated with bradykinin was confirmed in separate studies by chemiluminescence using the nitric oxide chemiluminescence analyzer (Warren JB, Wilson AJ, work in preparation), and also by bioassay using endothelial cells grown on microcarrier beads in a superfusion cascade system (O'Shaughnessy *et al.*, 1992).

To allow accumulation of endothelial-derived factors, superfusing buffer flow was halted 5 minutes before recordings were made. An indwelling thermocouple and heating element were used to maintain bath temperature and 95%O_2/5%CO_2 gas was superfused over the cell bath to maintain oxygenation and pH. The temperature of the system was controlled so that paired recordings were made at matched temperatures. Control studies showed that a change of +1.0°C from 32°C reduced contraction amplitude by 5±0.5% (mean±SEM, n=8), and similarly, a change of -1°C increased contraction by 5±0.5% (n=7). The complete data from the studies of superfusing buffer temperature are presented in chapter 3. For the recordings made during coculture experiments, bath temperature was maintained at 32.6±0.2°C. Some endothelium-myocyte studies were performed in the presence of 10^{-4}M N^G-nitro-L-arginine methyl ester (L-NAME) to inhibit production of nitric oxide, or in the presence of 10^{-5}M indomethacin to block prostaglandin synthesis.

Iloprost was a generous gift from Schering AG, Berlin, Germany.

6.1.3. **Results**

To examine the effect of endothelium-derived nitric oxide on myocyte contractility, myocytes were studied in primary coculture with confluent endothelium. To determine whether unstimulated endothelium affected baseline characteristics of contraction, the contractility of myocytes on endothelium was compared to that on glass. Contraction amplitude of myocytes adherent to quiescent endothelium ($5.3\pm0.6\%$ of resting length, $n=12$) was not different to myocytes adherent to glass coverslips ($5.3\pm0.3\%$, $n=32$). There was no difference in time to peak contraction (0.10 ± 0.02 s on endothelium, $n=11$; 0.12 ± 0.02 s on glass, $n=9$), nor relaxation time (0.30 ± 0.04 s on endothelium; 0.30 ± 0.03 s on glass).

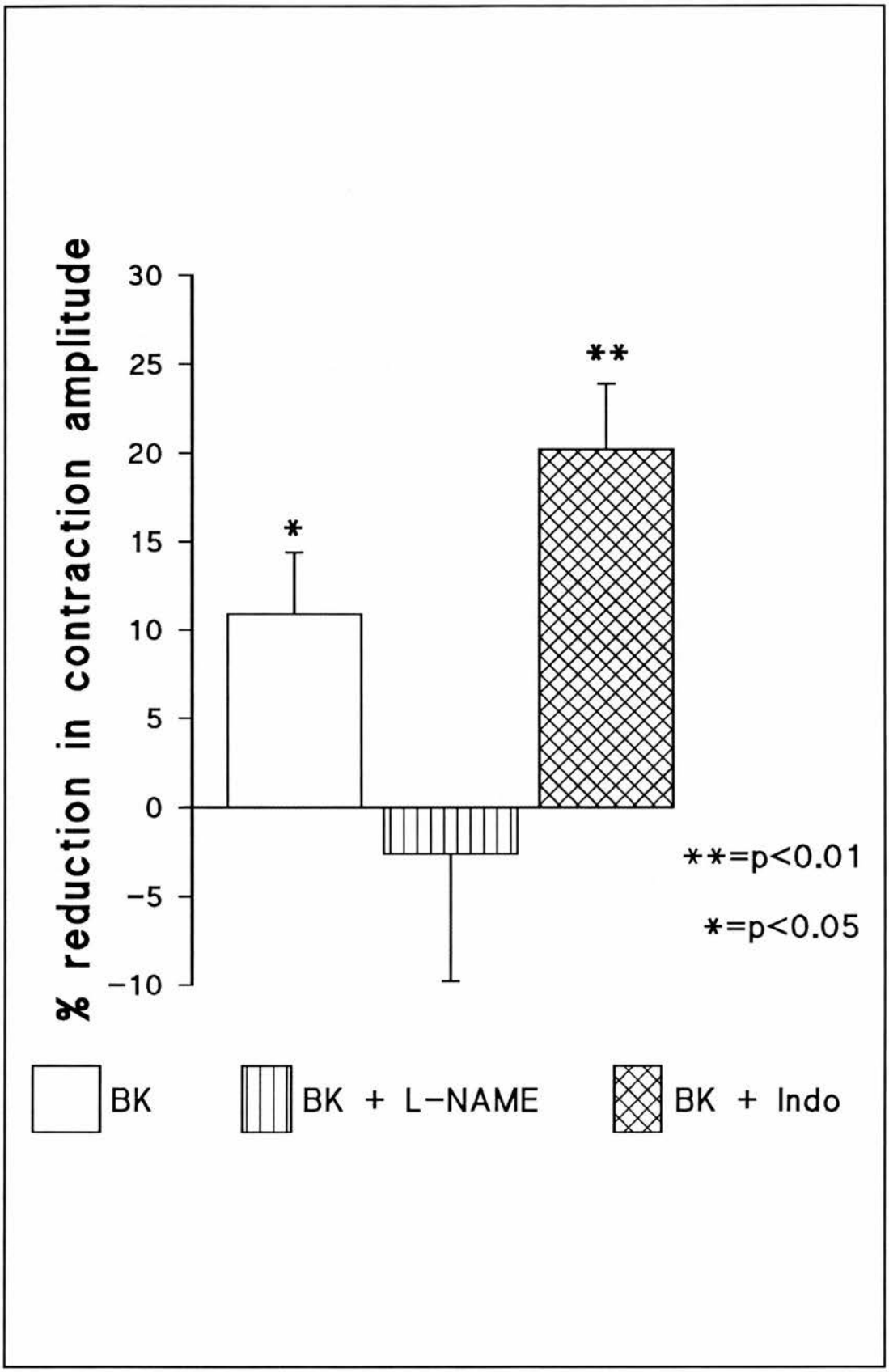


Figure 31 Endothelium-myocyte coculture; mean ± SEM of 12 (control ± BK), 11 (L-NAME ± BK) and 6 (indomethacin ± BK) experiments. BK, bradykinin; indo, indomethacin.

Myocyte contractility in coculture with endothelium.

In coculture experiments 10^{-7}M bradykinin was used to release nitric oxide from the endothelium. Myocyte contraction was reduced by $11 \pm 3.5\%$ ($p < 0.03$). This effect was abolished by the presence of 10^{-4}M L-NAME, and was not reduced by 10^{-5}M indomethacin (Figure 31). Although the bradykinin-induced reduction in contraction was greater in the presence of indomethacin, this further reduction was not significantly larger than that of bradykinin alone. Bradykinin-stimulated nitric oxide release had no effect on either time to peak contraction or relaxation time (see Table 5).

In control experiments without endothelium, bradykinin had no effect on contraction amplitude of isolated myocytes (Table 6). Bradykinin-stimulated nitric oxide release had no effect on either time to peak contraction (TTP), or time from peak contraction to 90% relaxation (PTR):

TTP:- 0.13 ± 0.01 s at baseline v 0.12 ± 0.01 s BK; n=6

PTR:- 0.39 ± 0.02 s at baseline v 0.38 ± 0.04 s BK; n=6

Even more importantly, L-NAME had no significant effect ($+1.0 \pm 3.6\%$ increase, n=9; p=NS, see Table 6 below) on contraction amplitude of normal ventricular myocytes without endothelium. This suggests that in their normal state, cardiac ventricular myocytes do not have appreciable nitric oxide synthase activity. L-NAME had no effect on time dependent variables either, as shown below:

TTP:- 0.11 ± 0.01 s at baseline v 0.11 ± 0.01 s L-NAME; n=9

PTR:- $0.29 \pm 0,02$ s at baseline v 0.27 ± 0.02 s L-NAME; n=9

Table 5 Effect of bradykinin, L-NAME and indomethacin on time to peak contraction and relaxation time of myocytes in coculture with endothelium.

	control	BK	10^{-4}M L-NAME	10^{-4}M L-NAME+BK	10^{-5}M indo	indo+BK
Time to peak contraction (s)	0.10±0.02	0.10±0.02	0.10±0.02	0.10±0.02	0.11±0.02	0.11±0.02
relaxation time (s)	0.30±0.04	0.28±0.03	0.30±0.02	0.28±0.03	0.38±0.03	0.34±0.03

Table 6 Effect of bradykinin, L-NAME and indomethacin on contraction amplitude of normal ventricular myocytes without endothelium. Mean±SEM of fractional shortening of contracting myocytes, expressed as a percentage of resting cell length.

	mean contraction amplitude±SEM at baseline	active agent: mean contraction amplitude±SEM	mean difference±SEM	mean difference as % of baseline value±SEM	p value
bradykinin 10^{-7}M; n=12	4.4±0.4%	4.3±0.4%	-0.1±0.3	+2.2±8.0%	NS
L-NAME 10^{-4}M; n=9	4.5±0.6%	4.7±0.79%	+0.2±0.2	+1.0±3.6%	NS
indomethacin 10^{-5}M; n=10	6.7±0.7%	6.4±0.7%	-0.3±0.2%	-5.0±3.7%	NS

Indomethacin had no effect on contraction amplitude in control experiments without endothelium (Table 6 above). In other controls without endothelium, Iloprost, the stable analogue of prostacyclin, had no effect on contractility over the dose range 10^{-7} -10^{-5}M, as shown.

Table 7 Effect of Iloprost 10^{-7}-10^{-5}M on contraction amplitude of control cardiac myocytes without endothelium. N=6 experiments at Iloprost $10^{-7}$$10^{-6}$M; n=5 at Iloprost $^{-5}$M.

	mean contraction amplitude±SEM at baseline	+Iloprost: mean contraction amplitude±SEM	mean difference as % of baseline value±SEM	p value
Iloprost 10^{-7}M	5.2±0.7%	5.2±0.7%	+1.6±2.7%	NS
Iloprost 10^{-6}M	4.5±0.2%	4.7±0.5%	+4.8±7.3%	NS
Iloprost 10^{-5}M	5.2±0.9%	5.5±1.1%	+3.0±12.2%	NS

6.1.4. **Discussion**

The contraction of cardiac myocytes was attenuated by nitric oxide released by endothelium. The contraction amplitude of myocytes was reduced by 11% when they were in close proximity to cultured endothelial cells which had been stimulated with bradykinin, a substance known to release nitric oxide from cultured bovine aortic endothelial cells. In control experiments bradykinin alone or non-stimulated endothelial cells had no effect on contractility. It is likely that the bradykinin-stimulated effect was mediated by the release of nitric oxide, or a similar intermediate, from the endothelial cells as it was blocked by the substrate inhibitor of nitric oxide synthase, L-NAME. The bradykinin-stimulated effect was independent of cyclooxygenase inhibition, excluding endothelial-derived prostaglandins as a cause of the attenuation. Neither indomethacin, the inhibitor of cyclooxygenase, not Iloprost, the stable analogue of prostacyclin, affected myocyte contractility in control experiments.

Stimulated endothelium had a lesser effect on contraction amplitude than did sodium nitroprusside or solution of nitric oxide, probably because the local

concentration of nitric oxide generated within the myocyte from the nitroprusside or directly administered was higher than that made available by exogenous stimulation of endothelium.

L-NAME had no effect on contractility of isolated cardiac myocytes in the absence of endothelium. This was an important control study because it shows that ventricular myocytes from healthy guinea-pigs do not possess intrinsic nitric oxide synthase activity under normal circumstances. It also shows that L-NAME has no other effects on contractile apparatus. Another specific nitric oxide synthase inhibitor, L-NMMA was shown to have no effect on contractility of normal rat cardiac myocytes (Amrani *et al.*, 1992). Constitutive nitric oxide synthase has now been shown to be present in normal, healthy cardiac myocytes (Schultz *et al.*, 1992; Balligand *et al.*, 1993) but does not appear to generate appreciable nitric oxide. Inhibitors and substrates of nitric oxide synthase had no effect on contractile function of cells in their basal state, either in my experiments (this chapter, and Chapter 7) or theirs (Amrani *et al.*, 1992; Schultz *et al.*, 1992; Balligand *et al.*, 1993).

My hypothesis that important interactions exist between endothelium and myocardium is supported by studies that have shown influence of endocardial endothelium over adjacent myocardial cells in papillary muscle experiments *in vitro*. This was first described by Brutsaert and coworkers who showed that damage to the endocardial surface of papillary muscle causes a reduction of about 20% (at 2.5 mM external Ca^{2+} concentration) in maximum isometric tension produced by the muscle, brought about by an earlier onset of isometric relaxation, but with no effect on the velocity of contraction (Brutsaert *et al.*, 1988). This effect occurs in endocardium-intact preparations with administration of lipid-soluble analogues of cGMP, or with sodium nitroprusside and atrial natriuretic peptide (substances that increase myocardial cGMP by stimulating soluble and particulate guanylate cyclase respectively), which reduce papillary muscle contractility similar to endocardial damage(Smith *et al.*, 1991; Brutsaert *et al.*, 1988; Shah & Henderson, 1992). From experiments using cultured endocardial cells on beads in a cascade system, endocardial endothelium releases a substance indistinguishable from endothelium-derived nitric oxide with negative inotropic effects, and an as yet unidentified opposing substance which both augments contraction and prolongs relaxation (Smith *et al.*, 1991). Restoration of

endocardial products to endocardium-damaged papillary muscles both increases the force of isometric contraction and prolongs the relaxation phase after contraction in this system. The quantitative relationship between this inotropic endocardial product and endocardial nitric oxide is yet to be fully established, although the unidentified contraction-prolonging factor appeared to be dominant in these experiments (Shah & Henderson, 1992).

Another group examined histochemical evidence of contractile activity in blocks of myocardium or in papillary muscles (McClellan *et al.*, 1992). They found an unequal distribution of myosin ATPase activity in myocardium, with activity highest in those cells nearest blood vessels. Their explanation was that factor(s) released from blood vessels modified myosin ATPase activity, although they could not determine what these factors were.

Implicit in the hypothesis for the present study is that coronary microvascular endothelium releases nitric oxide, but there is yet no direct evidence that this occurs. I used non-enzymatically cultured bovine aortic endothelium for the cardiac myocyte-endothelium coculture experiments since it is both readily obtained and is a known source of nitric oxide. But important differences exist between the endothelium lining large arteries, and endothelium within the microvasculature. For example, coronary microvessels are less sensitive than aorta to the exogenous nitrovasodilator, GTN, but relax equally to nitric oxide solution, albeit at a higher concentration (Sellke *et al.*, 1990). This implies that metabolism of organic nitrates in the coronary microvasculature is less efficient than elsewhere in the circulation, perhaps because of a relative deficiency of either the enzymes required (Chung & Fung, 1990), or available sulfhydryl groups necessary for generation of nitric oxide from GTN. Nitric oxide has been shown to be a vasodilator in several different microvascular preparations (Warren *et al.*, 1992; Gardiner *et al.*, 1990; Ekelund & Mellander, 1990; Persson *et al.*, 1990).

It has been demonstrated recently in isolated, perfused hearts that 5-hydroxytryptamine stimulates nitric oxide release from the coronary microvasculature, measured by coronary sinus nitrite sampling, and this is accompanied by a reduction in the time to reach peak ventricular pressure (Shah *et al.*, 1991). These preliminary data suggest that coronary microvascular endothelium, as well as the endocardial

endothelium covering papillary muscles, is capable of releasing factors which modulate myocardial contraction.

Support for a functional relationship between nitric oxide and the myocardium comes from three recent reports. Finkel et al (Finkel *et al.*, 1992) have shown that the negative inotropic effects of inflammatory cytokines on papillary muscle are mediated rapidly by generation of nitric oxide within the muscle itself. Such effects in other tissues require *de novo* protein synthesis and the rapid time course of their results has been questioned. Moncada's group have demonstrated the presence of both the constitutive and inducible forms of the nitric oxide synthase enzyme in rat cardiac myocytes (Schulz *et al.*, 1992), and I have shown recently that in experimental endotoxemia cardiac myocyte contractility is markedly impaired by a mechanism involving nitric oxide production within the myocytes themselves (Brady *et al.*, 1992). Thus cardiac myocytes contain the biochemical apparatus necessary to both generate and handle nitric oxide. My evidence that healthy cardiac myocytes are sensitive to exogenous nitric oxide in three different delivery systems supports the hypothesis that microvascular endothelium influences cardiac myocyte contraction by activation of nitric oxide-sensitive guanylate cyclase, although nitric oxide production by microvascular endothelium within the heart has yet to be demonstrated.

The mechanism by which nitric oxide may cause a reduction in myocyte contraction is not understood. In chapter 5 I discussed possible mechanisms of cGMP action within myocardium. An alternative, although less likely explanation of the effect of nitric oxide on cardiac myocytes is that the elasticity of proteins forming the cytoskeleton, rather than the actin-myosin myofibrils, is modified by nitric oxide. Important changes in cytoskeletal proteins occur in ischaemia (Steenbergen *et al.*, 1987a) and in cardiomyopathy (Schaper *et al.*, 1991), but these changes probably evolve over a long time course. Whether nitric oxide can cause transient changes in cellular ultrastructure has not been described.

Recently, L-NAME has been shown in rabbit coronary artery to have actions of a muscarinic antagonist (Buxton *et al.*, 1993) as well as its inhibitory effect on nitric oxide synthase. L-NMMA does not have this property. This study has important bearing on many reports, but since the only action of L-NAME in this chapter and the next was a specific inhibition of nitric oxide synthesis, the

interpretation of my results is not affected by these implications.

That nitric oxide affects cardiac myocyte contractility may have important consequences. It may be that in normal hearts, capillary endothelium produces EDRF which has a tonic effect on contraction of nearby myocytes. With the development of cardiac hypertrophy or dilatation in disease states, the normal architecture and relationship between microvasculature and myocytes becomes disturbed with increased diffusion distance and impairment of function. Implications of this research are discussed further in chapter 11.

Further new evidence of a role of nitric oxide modulating cardiac myocyte contraction has recently been published by Michel's group in Boston (Balligand *et al.*, 1993). Studies were performed in both neonatal and adult ventricular myocytes. Constitutive nitric oxide production by healthy cells of both types was demonstrated, although neither the provision of substrate, L-arginine, nor the presence of the inhibitor, LNMMA, had an effect on basal contractility. Nitric oxide activity was demonstrated as a modulating influence on ß-adrenergic stimulated inotropy in adult cells, and nitric oxide appeared to mediate the effect of the muscarinic agonist, carbachol, on myocytes. LNMMA augmented isoprenaline-induced contraction, and LNMMA blocked the attenuating effect of carbachol on myocyte contraction.

8-bromo-cyclic GMP had the same effect as nitric oxide in their studies, and methylene blue and oxyhaemoglobin reversed the moderating influence of nitric oxide. Interestingly, sodium nitroprusside did not restore the negative inotropic effect of LNMMA on carbachol-treated myocytes. Sodium nitroprusside did not attenuate contractility in their experiments, although they did not formally examine the effects of sodium nitroprusside directly ($n=3$ in their controls). A possible explanation may be that signal transduction mechanisms mediating the effect of carbachol involve more than production of free nitric oxide within cells.

CHAPTER 7: EFFECTS OF ENDOTOXIN TREATMENT ON CARDIAC MYOCYTE CONTRACTILITY

7.1.1. **Introduction**

While endothelial control of vascular tone is central to normal cardiovascular function, disturbances of nitric oxide physiology have recently been shown to occur in disease states. One such example is endotoxic shock. Septicaemia and accompanying septic shock account for a substantial number of hospital deaths, despite appropriate antibiotic and supportive therapy. In the earliest stages of septic shock stroke volume and cardiac output are maintained or even increased; later, ventricular dilatation develops with a reduction in ejection fraction (MacLean *et al.*, 1967; Parker *et al.*, 1984; Ellrodt *et al.*, 1985). If patients survive, ventricular size and function return to normal as the infection is controlled and circulatory function restored.

In endotoxic shock the presence of disseminated foreign antigen, together with the inflammatory response, causes an inducible nitric oxide synthase to be generated in many cell types which do not normally express this enzyme, including hepatocytes, fibroblasts and vascular smooth muscle (Moncada *et al.*, 1991; Nathan, 1992). Subsequent production of large quantities of nitric oxide leads not only to haemodynamic instability, but also to widespread production of nitric oxide-based free radicals which have the potential to cause considerable damage to tissues.

Vascular smooth muscle is itself not a source of nitric oxide in health. In endotoxic shock the production of nitric oxide occurs within the muscle layer of the vessel wall causes excessive vasodilatation and hence a reduction in peripheral vascular resistance (Moncada *et al.*, 1991). Substrate inhibitors of nitric oxide synthase cause systemic vasoconstriction and a pressor response in healthy animals by inhibiting constitutive nitric oxide production by the endothelium. In animals with experimental endotoxic shock inhibitors of nitric oxide synthase reverse hypotension, but also cause a sustained increase in systemic vascular resistance and at higher doses a decrease in cardiac output (Klabunde & Ritger, 1991; Nava *et al.*, 1991; Wright *et al.*, 1992). A reduction in cardiac output has also been seen with these agents in

normal animals (Klabunde & Ritger, 1991; Kilbourn *et al.*, 1990; Aisaka *et al.*, 1989). Whether this fall in cardiac output is secondary to the rise in vascular resistance, or to a direct effect of nitric oxide synthase inhibitors on cardiac contractility, was not addressed by these studies.

Global deterioration of myocardial contractile function in patients with endotoxic shock has been established by clinical and radionuclide studies (MacLean *et al.*, 1967; Parker *et al.*, 1984; Ellrodt *et al.*, 1985). In normal volunteers administration of purified endotoxin causes reversible depression of left ventricular function, in addition to the expected reduction in systemic vascular resistance (Suffredini *et al.*, 1989). Until recently, the cause of myocardial depression in endotoxaemia was considered to be a direct effect of endotoxin or an inflammatory mediator on myocardial tissue. The existence of a specific circulating myocardial depressant substance in endotoxic shock has been postulated, but not proven (Ellrodt *et al.*, 1985). While coronary perfusion abnormalities in patients with coexisting cardiac or coronary disease and endotoxic shock may account for segmental abnormalities of left ventricular function, in patients with global myocardial impairment and endotoxic shock the loss of function cannot be wholly explained by changes in coronary flow (Ellrodt *et al.*, 1985). As in the peripheral vasculature, multiple factors exist which depress cardiac function in endotoxic shock. However, there may be a common pathway for such mediators to impair myocardial contraction.

My hypothesis tested in these experiments is that, similar to the behaviour of vascular smooth muscle in endotoxic shock, production of nitric oxide occurs within cardiac myocytes in this condition and contributes to the reduced cardiac contractility seen in endotoxaemia. This study was designed to examine whether contractility of cardiac myocytes was reduced by endotoxin treatment of animals, and if so, whether inhibition of the synthesis or transduction of nitric oxide would modify this attenuation.

7.1.2. **Methods**

Endotoxic shock model

Animals treated with endotoxin (lipopolysaccharide, *E. coli* serotype 055:B5; Sigma, Poole, Dorset, UK) were administered 4 mg/kg intraperitoneally, 4 h before sacrifice. In some cases, an intravenous injection of dexamethasone 4mg/kg (David Bull, Warwick, UK) was given 1 h before injection of endotoxin. Cardiac ventricular myocytes were isolated by enzymatic digestion, as before.

Once stable contraction had been achieved, myocytes were exposed for 10 min to nitric oxide synthase inhibitors and substrates, and values obtained were compared to the mean of control contractions measured both before and after exposure to each drug. All recordings were made within 45 min of stabilisation to avoid the decline in myocyte contraction amplitude which is seen in experiments lasting longer than 2 hours. Some experiments were performed in the presence of the guanylate cyclase inhibitor, methylene blue (David Bull Laboratories). In these experiments, recordings were made after 15 min exposure, and compared to baseline before administration. The nitric oxide synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-methyl-L-arginine (L-NMMA), and substrates L-arginine and D-arginine, were obtained from Sigma.

7.1.3. **Results**

Effect of endotoxin treatment on contraction amplitude of cardiac myocytes.

L-NAME had no effect on contractility of myocytes from normal animals (see chapter 6). This suggests that there is no nitric oxide synthase activity in cardiac ventricular myocytes in health. The second series of studies examined the contraction of myocytes from guinea pigs with experimental endotoxaemia. Figure 32 shows the effect of endotoxin (lipopolysaccharide) treatment on myocyte shortening. *This study generated the most important finding of the entire thesis: contraction amplitude of myocytes isolated from endotoxin treated animals was reduced by 46% ($p<0.001$) when compared to cells from control animals. The reduced contractility of myocytes from endotoxin treated animals was substantially reversed by the nitric oxide synthase inhibitor L-NAME, whereas L-NAME had no effect on control cells. Pre-treatment with dexamethasone, known to block induction of inducible nitric oxide synthase, abolished the effects of endotoxin.* Addition of L-NAME to cells from animals treated with both dexamethasone and endotoxin had no further effect (fractional shortening $5.0\pm0.9\%$; $n=7$, $p=$NS, compared to normal cells).

Effect of substrates and inhibitors of nitric oxide on contraction amplitude of myocytes from endotoxin treated animals.

Figure 33 shows the effects of different substrates and inhibitors on contraction of myocytes from endotoxin-treated guinea-pigs. Addition of 10^{-4}M L-NAME to myocytes from endotoxin-treated animals increased contraction amplitude by $40\pm6.6\%$ ($p<0.001$, $n=17$). Contractility returned to baseline within a 10 min washout period. N^G-methyl-L-arginine (L-NMMA) gave similar results. The effect of L-NAME was reversed by coadministration of 10^{-3}M L-arginine, but not D-arginine. Neither amino acid had a significant effect on control myocytes (Table 10). L-NAME 10^{-4}M had no effect on contraction of normal myocytes isolated from control animals (Table 10 and chapter 6).

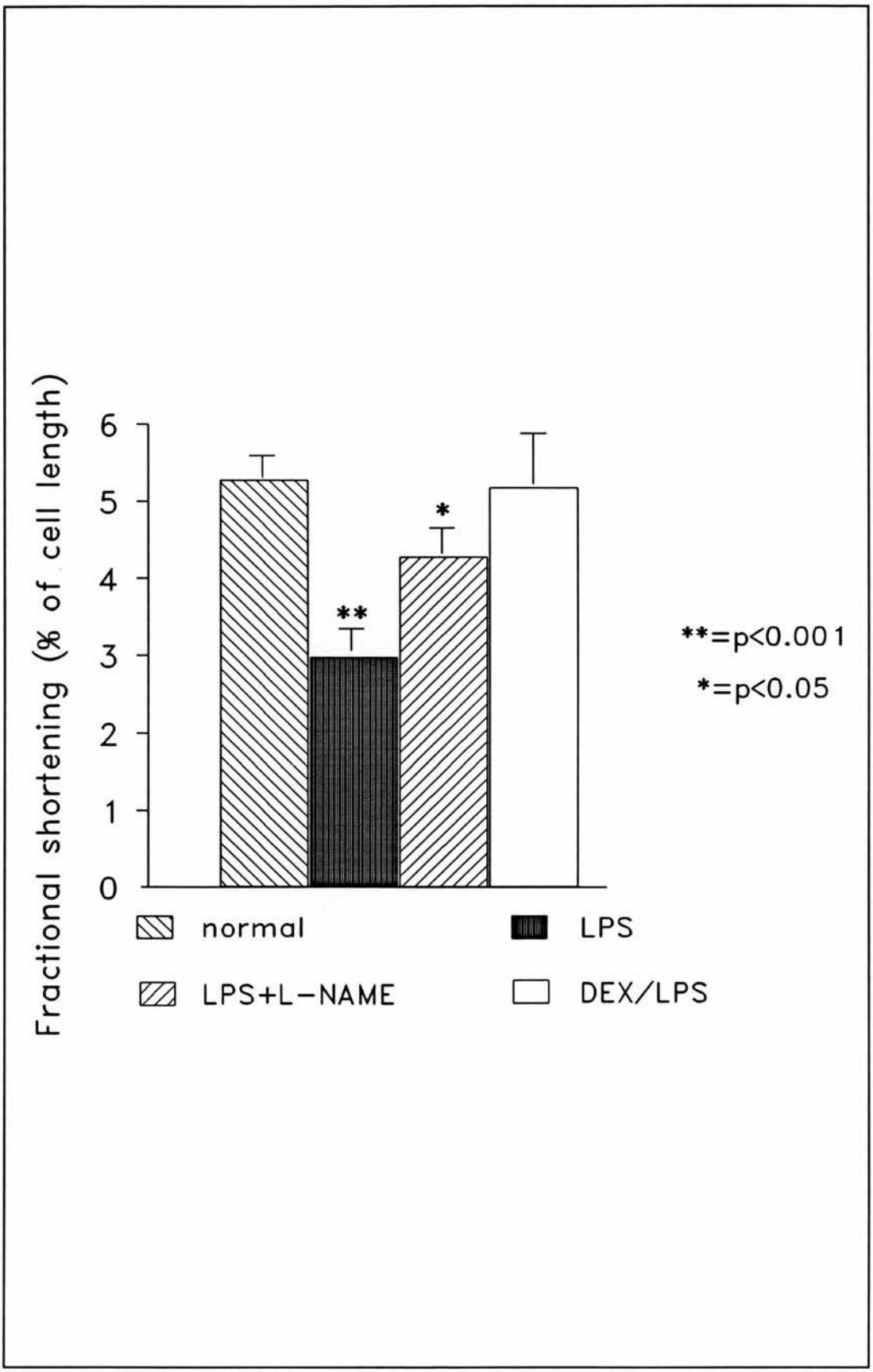


Figure 32 Effect of endotoxin (lipopolysaccharide, LPS) treatment on fractional shortening. Dex, dexamethasone. Mean ± SE of 32 (normal), 17 (LPS treated) and 7 (LPS + dexamethasone) experiments.

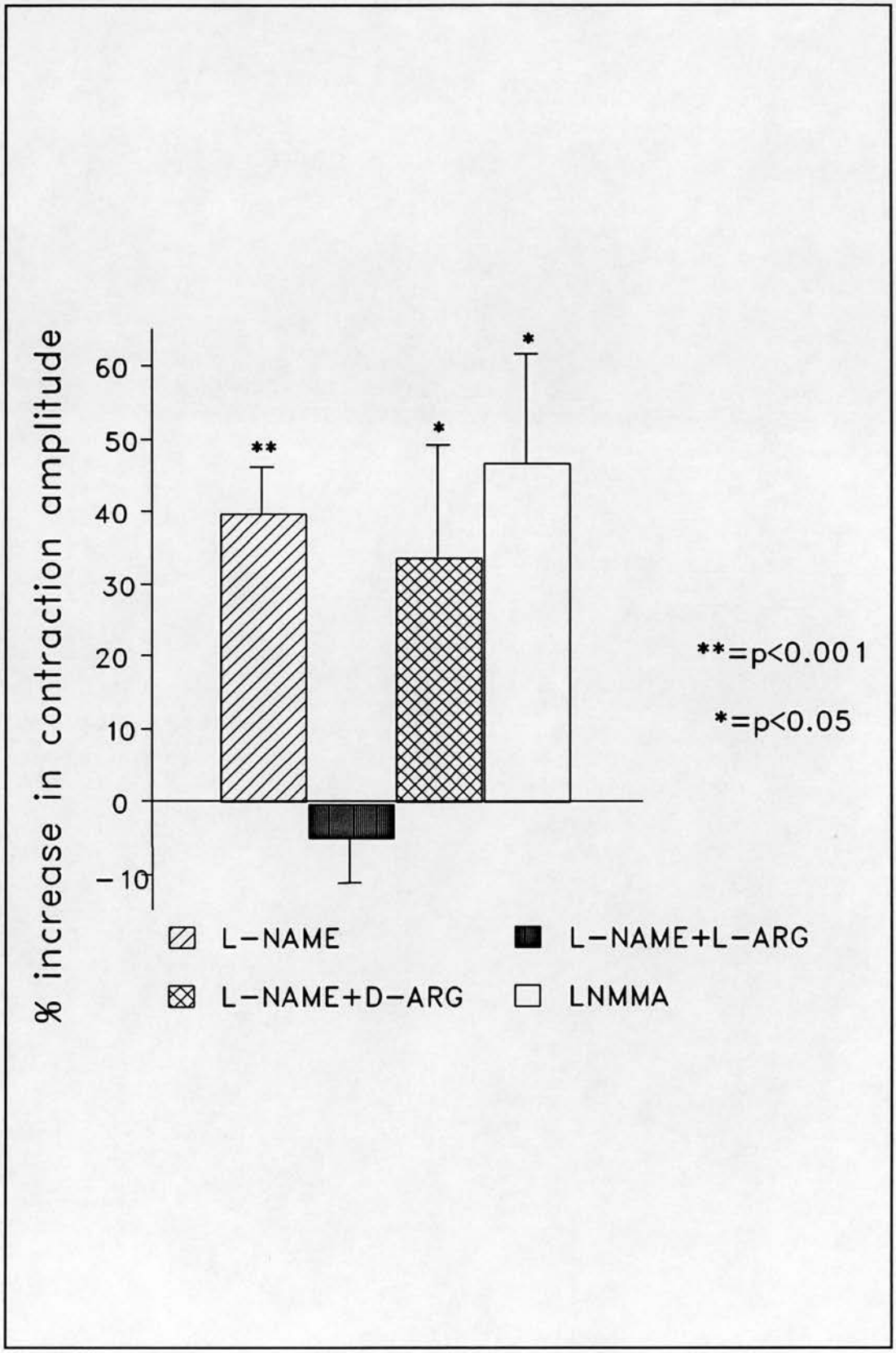


Figure 33 Effect of substrates and inhibitors of NO synthase on contractility of myocytes from endotoxin-treated animals. n = 17 (L-NAME), 8 (L-arg + L-NAME), 6 (D-arg + L-NAME) and 5 (L-NMMA) experiments.

Table 8 Endotoxin treatment had no effect on either time to peak contraction (TTP) or 90% relaxation time (PTR) compared to myocytes from normal animals:

	Normal myocytes; n=9	Myocytes from endotoxin-treated animals; n=17	p value
Time to peak contraction (TTP)	0.12±0.02s	0.10±0.02s	NS
Relaxation time (PTR)	0.30±0.03s	0.32±0.02s	NS

Table 9 Addition of L-NAME to myocytes from endotoxin treated guinea pigs increased contraction amplitude substantially but had no effect on TTP/PTR:

Myocytes from endotoxin-treated animals; n=17	baseline	+ L-NAME	p value
Time to peak contraction (TTP)	0.10±0.02s	0.10±0.01s	NS
Relaxation time (PTR)	0.32±0.02s	0.35±0.02s	NS

Dexamethasone pretreatment of healthy guinea pigs had no effect on myocyte contraction in control studies (see Table 10).

Effect of methylene blue on contraction amplitude of myocytes from normal and endotoxin-treated animals.

Exposure of cells from endotoxin-treated animals to methylene blue $5x10^{-6}$M for 15 min increased contraction amplitude substantially, by 157±27% (fractional shortening increased from 2.3±0.4% of cell length to 6.1±1.4% of cell length; n=7, $p<0.005$), as shown in Figure 34. Exposure of normal control myocytes to methylene blue also increased contraction amplitude at 15 min (by 54±11%, n=8, $p<0.01$). Interestingly, the maximum contraction of cells from both normal and

endotoxin-treated animals in the presence of methylene blue was similar. In experiments with myocytes from both normal and endotoxin treated animals, some cells showed signs characteristic of calcium overload, with hypercontractility and fibrillation, after 20 min superfusion with methylene blue. Four of seven myocytes from endotoxin treated animals, and four of eight normal myocytes, failed to return to baseline contraction after exposure to methylene blue.

Table 10 Effect of substrates and inhibitors of nitric oxide synthase on contraction amplitude of normal ventricular myocytes. Mean ± SEM of fractional shortening of contracting myocytes, expressed as a percentage of resting cell length.

	mean contraction amplitude ± SEM at baseline	active agent: mean contraction amplitude ± SEM	mean difference ± SEM	mean difference as % of baseline value ± SEM	p value
L-NAME 10^{-4}M; n=9	4.5±0.6%	4.7±0.79%	+0.2±0.2	+1.0±3.6%	NS
L-arginine 10^{-3}M; n=5	3.2±0.4%	3.3±0.5%	+0.1±0.2	+3.9±4.7%	NS
D-arginine 10^{-3}M; n=5	3.2±0.5%	3.3±0.5%	+0.1±0.1	+4.2±3.6%	NS
dexamethasone 4mg/kg pretreatment; n=11	5.3±0.5%; n=12 normal animals	5.0±0.4% dexamethasone treated animals			NS

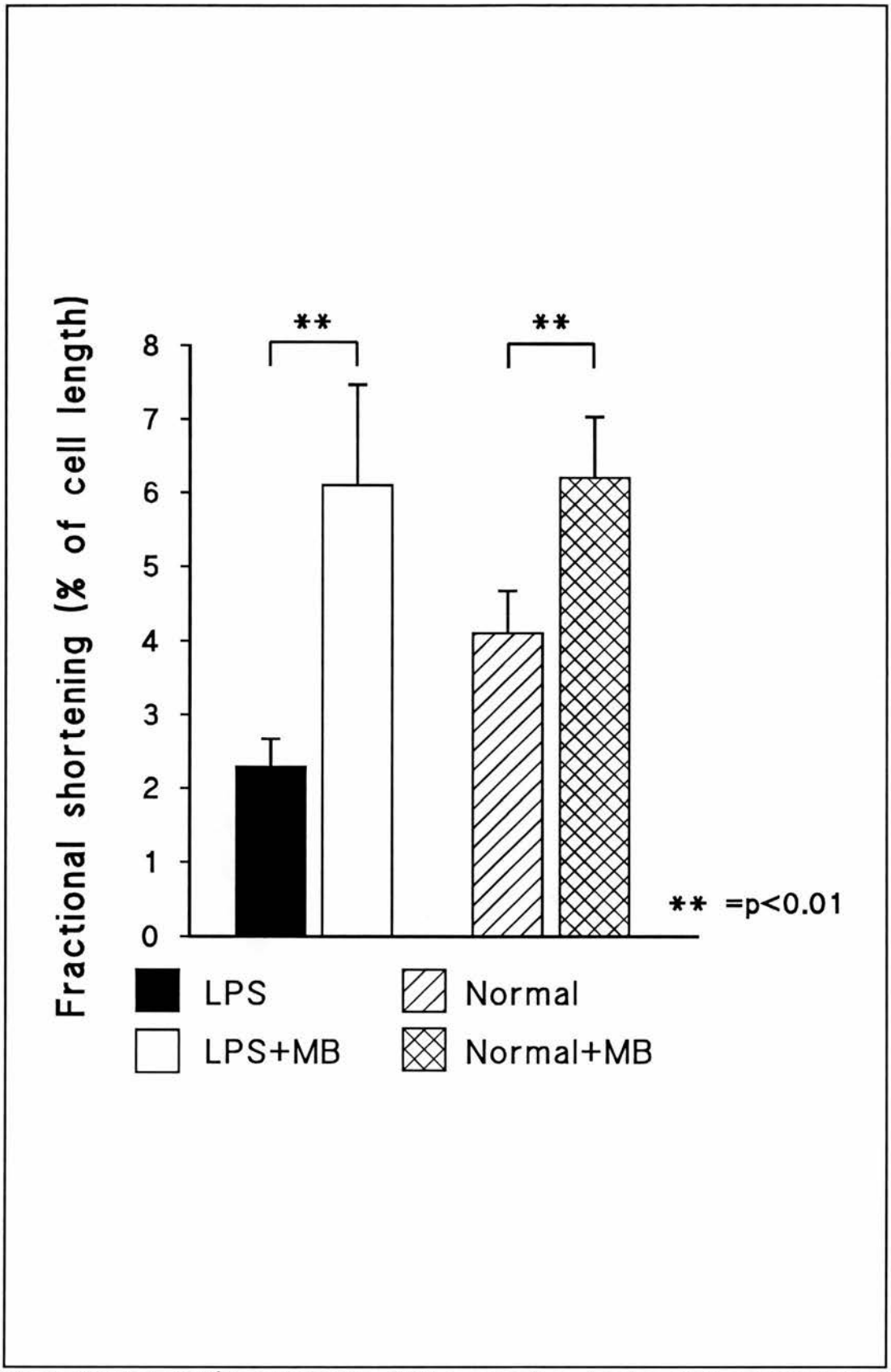


Figure 34 Effect of 10^{-5}M methylene blue (MB) on myocyte contraction. LPS, myocytes isolated from endotoxin-treated animals; normal, myocytes from healthy animals.

7.1.4. **Discussion**

Endogenous production of nitric oxide within cardiac myocytes contributes to their reduced contractility in endotoxaemia.

I have shown that endotoxin treatment of guinea-pigs causes a substantial reduction in the contractility of isolated cardiac myocytes. About half of this reduction can be reversed by specific nitric oxide synthase inhibitors, and their effect can be overcome by an excess of L-arginine, the L-isomer amino acid from which nitric oxide is generated, but not by the inactive D-isomer. These results suggest that nitric oxide is being produced within the myocytes themselves, and that this nitric oxide has an attenuating effect on myocyte contraction.

As I was completing these experiments, biochemical evidence became available of the presence of nitric oxide synthase enzymes in myocardium (Schulz *et al.*, 1992). In that study, endotoxin caused an elevation in cGMP, and pretreatment with dexamethasone prevented the induction of inducible nitric oxide synthase within cardiac myocytes. In the present study prior administration of dexamethasone prevented the reduction in contractility caused by endotoxin, and subsequent addition of L-NAME had no effect. This is further evidence for inducible nitric oxide synthase activity within the heart contributing substantially to the reduction in contractility occurring in endotoxic shock. Normal cardiac myocytes contain detectable amounts of constitutive nitric oxide synthase (Schulz *et al.*, 1992), although this does not appear to generate effective levels of nitric oxide, since in the present study, and in the first series of experiments nitric oxide synthase inhibitors had no effect on contractility of cardiac myocytes isolated from healthy animals.

Nitric oxide synthase inhibitors did not completely reverse the effect of endotoxin. This suggests that factors other than nitric oxide might contribute, although it is possible that nitric oxide production was not blocked completely by the nitric oxide synthase inhibitors. While endotoxin may itself be cytotoxic to myocytes many other inflammatory mediators are released in endotoxic shock. Recent attention has focused on the use of anti-tumour necrosis factor (TNF) antibodies in septic shock, which appears to improve left ventricular function in such patients (Vincent *et al.*, 1992). In the present study the effect of endotoxin was abolished by pre-

treatment with dexamethasone. Steroids not only prevent the induction of nitric oxide synthase by endotoxin but also prevent the generation of many inflammatory cytokines which might impair myocyte contractility. Such cytokines may themselves act by induction of nitric oxide synthesis within the heart: in a recent study the cytokines TNFα, interleukin-2 and interleukin-6 inhibited myocardial papillary muscle contraction by a nitric oxide dependent mechanism (Finkel *et al.*, 1992), although the rapid time course of this study suggests activation of an existing constitutive nitric oxide synthase enzyme, rather than induction of new protein. Neonatal cardiac myocytes cultured in a mixture of IL-1 and TNFα show depressed responses to ß-adrenergic stimulation (Gulick *et al.*, 1989), although in this study the role of nitric oxide was not considered.

Inhibition of inducible nitric oxide synthase may have therapeutic potential in patients with endotoxic shock. In animal models of endotoxic shock, recent studies have shown that nitric oxide-synthase inhibitors were without significant haemodynamic benefit given in small doses whereas larger doses caused intense peripheral vasoconstriction and cardiovascular collapse (Nava *et al.*, 1991; Wright *et al.*, 1992; Klabunde & Ritger, 1991). It appears that at high doses inhibition of both the normal, constitutive nitric oxide synthase and the inducible nitric oxide synthase occurs, and complete inhibition of endogenous nitric oxide synthesis then allows unopposed peripheral vasoconstriction, deleterious in endotoxic shock. Either coadministration of a nitric oxide donor with a nitric oxide synthase inhibitor, or selective inhibition of the inducible form of nitric oxide synthase might be of benefit in this condition (Wright *et al.*, 1992).

Action of methylene blue on contractility of myocytes from endotoxin treated animals.

Over the short time course of the experiments on healthy cardiac myocytes described in chapter 4, methylene blue reversed the negative inotropic effects of nitric oxide from sodium nitroprusside. However, superfusion with methylene blue for prolonged periods >30-60 min caused these cells to contract much more strongly, before fibrillating. Exposure of myocytes to methylene blue in the present study caused a large increase in contraction amplitude of cells from endotoxin-treated animals. Contraction of normal myocytes was also increased, although the time

course of these studies was longer than the early experiments with sodium nitroprusside. In both series of studies administration of methylene blue caused some of the myocytes to become hypercontractile, with contractions characteristic of calcium overload. It may be that exposure to methylene blue causes progressive injury to cardiac myocytes, perhaps by increasing the permeability of the cells to extracellular calcium. The explanation why contractility of some of the cells did not return to baseline when methylene blue superfusion was discontinued is not clear. Whether the effect of methylene blue is limited to changes in cGMP concentration, or whether methylene blue has a direct toxic effect, is not explained by either study.

The present study is the first to show direct loss of contractility of cardiac myocytes in endotoxic shock, associated with the induction of nitric oxide synthase and production of nitric oxide within the myocytes. Contractility was restored partially by nitric oxide synthase inhibitors, in keeping with two recent clinical case reports. In two patients with septic shock, administration of L-arginine analogues caused short-term increases in blood pressure. Cardiac output was increased in one patient and decreased in the other (Petros *et al.*, 1991). A further intriguing report described short-lived improvement in a patient with hepatic failure following administration of methylene blue (Midgley *et al.*, 1991). Methylene blue inhibits guanylate cyclase, but does not inhibit the cytotoxic effects of nitric oxide. In the present study I found methylene blue to be toxic to myocytes after prolonged administration, although the reasons for this effect are not yet clear, as described above. The loss of contractility after endotoxin exposure can be prevented by pretreatment of animals with dexamethasone, but it is generally accepted that steroid therapy is of no benefit to patients with endotoxic shock (Bone *et al.*, 1987; Sprung *et al.*, 1984; The Veterans Administration Systemic Sepsis Cooperative Study Group, 1987). Until a specific inhibitor of inducible nitric oxide synthase is available, careful titration of a nitric oxide synthase inhibitor, perhaps combined with a nitric oxide donor, may be a possible treatment in endotoxic shock, not only by reducing the profound peripheral vasodilatation (Wright *et al.*, 1992), but also by augmenting myocardial contraction. This is discussed further in the General Discussion in Chapter 11.

CHAPTER 8: CARDIAC MYOCYTE CONTRACTILITY AFTER ISCHAEMIA AND REPERFUSION

8.1.1. **Introduction**

Short periods of myocardial ischaemia are accompanied by a transient reduction in contractile function. This was first described in 1975 by Heyndrickx (Heyndrickx *et al.*, 1975) and is now popularly termed myocardial "stunning" (Braunwald & Kloner, 1985). Central to the definition of stunning is that despite restoration of coronary flow after a transient occlusion, mechanical dysfunction persists, but this dysfunction ultimately recovers (Bolli, 1992). This means that there is mechanical dysfunction without irreversible injury. The time course of recovery from myocardial stunning is related to the duration of ischaemia. After 1 min of coronary occlusion active shortening in the affected area returns within 20 s, and is complete by 30 min (Braunwald & Kloner, 1985). Following a single 15 min episode of myocardial ischaemia in dogs slight return of function is seen 5 min into the reperfusion period, although even after 1 h of reperfusion contractility has reached only 50% of baseline. Function has not quite recovered by 24 h but reaches normal by 48 h (Bolli *et al.*, 1988).

Even after prolonged ischaemia, some return of myocardial function in the affected area may be seen as late as 1-2 weeks after the event (Braunwald & Kloner, 1985). Ischaemic myocardial necrosis must not be confused with stunning, since stunning is fully reversible whereas infarction is not. For laboratory studies of reperfusion injury, periods of ischaemia of 30-90 min are usually employed so that a moderate degree of myocardial injury is achieved, whereas to induce reversible stunning of myocardium coronary occlusion of up to 20 min is performed (Heyndrickx *et al.*, 1975).

8.1.2. **Proposed mechanisms of myocardial stunning**

There have been different hypotheses to try to explain the myocardial dysfunction of stunning. Stunned myocardium can maintain ATP synthesis in the face

of increased demand, therefore inadequacy of energy production is not the explanation (Ellis *et al.*, 1984). Abnormalities of coronary vascular function occur in stunning. While prolonged ischaemia, severe enough to cause myocyte necrosis, is associated with endothelial cell swelling and injury (Kloner *et al.*, 1974; Piper *et al.*, 1990) there is accumulating evidence that important alterations of endothelial function occur much earlier in ischaemia, before evidence of morphological damage appears. Brief periods of ischaemia cause loss of endothelium-dependent vasodilatation and enhanced coronary vasoconstrictor responses (Kim *et al.*, 1992), together with increased permeability of the coronary endothelium (Dauber *et al.*, 1990). These functional changes occur without evidence of structural injury. There is evidence of injury caused by oxygen radicals in ischaemic-reperfusion injury (see sections 1.6.5) and oxygen radical scavengers attenuate the endothelial dysfunction in stunned myocardium (Bolli, 1992). As well as being a target for free radical-mediated damage, endothelium itself generates these species in reperfusion injury (Zweier *et al.*, 1988; Schinetti *et al.*, 1989). The commonly used agents catalase and superoxide dismutase, and "spin trap" agents (drugs which bind and inactivate free radicals) are large molecules which do not readily cross cell membranes (Downey & Yellon, 1992). One explanation of their beneficial action in the recovery from myocardial stunning is that they bind endothelial-derived free radicals and thus reduce exposure of myocytes to these injurious species (Mullane & Young, 1992). Another possibility is that free radicals produced within myocytes will pass through the cell membrane down a concentration gradient. Continuously "mopping up" these radicals outside the cell by scavengers may aid myocyte recovery.

Bolli has listed four likely explanations of myocardial stunning (lecture to National Heart and Lung Institute, London, 19th October 1992; also reference- (Bolli, 1992). These are:

(i) Reduced sensitivity of myofilaments to calcium

(ii) Transient calcium overload within myocytes

(iii) Excitation-contraction uncoupling due to sarcoplasmic reticulum dysfunction

(iv) Generation of oxygen free radicals

These are not mutually exclusive, but are discussed individually.

Reduced sensitivity of myofilaments to calcium

Stunned isolated hearts are less sensitive to increased levels of extracellular calcium (Kusuoka *et al.*, 1987). Cytosolic free calcium is not reduced (Kusuoka *et al.*, 1990) and one suggestion is that the myofilaments become less sensitive to calcium. But stunned myocardium *in vivo* responds normally to inotropic agents, as mentioned above, and this means that myofilament handling of calcium is not impaired under these conditions. Furthermore, in single cells myofilament relaxation is impaired following hypoxia (Silverman *et al.*, 1991). The time course of this disturbance is not matched by alterations in $[Ca^{2+}]_i$. The mechanism of altered relaxation is thus not readily explained by changes in $[Ca^{2+}]_i$. Silverman (Silverman *et al.*, 1991) suggested that abnormalities of phosphorylation of myofilaments might explain their results.

Excitation-contraction uncoupling due to sarcoplasmic reticulum dysfunction

Injury to the sarcoplasmic reticulum with impaired release of calcium to the contractile proteins has been suggested (Krause *et al.*, 1989) and by itself might contribute to the reduced contractility of stunned myocardium. Other workers have shown no reduction in $[Ca^{2+}]_i$ following ischaemia (Kusuoka *et al.*, 1990), although these conflicting ideas could be reconciled by alterations in compartmentalisation of Ca^{2+} within the cell.

Transient calcium overload within myocytes

Intracellular $[Ca^{2+}]$ is elevated in ischaemic myocardium [Steenbergen #31]. Reperfusion with low Ca^{2+} solution protects myocardium after an ischaemic episode (Kusuoka *et al.*, 1987). Cardiac myocyte hypercontracture following reperfusion after hypoxia is characterised by a sudden further rise in $[Ca^{2+}]_i$ (Josephson *et al.*, 1991). But intracellular $[Ca^{2+}]$ is only elevated in stunned reperfused myocardium during the first few minutes following restoration of flow (Bolli, 1992), while

mechanical dysfunction is prolonged. The mechanism of this elevation of $[Ca^{2+}]_i$ is under intensive study. There is impairment of Na^+-Ca^{2+} exchange, Na^+-K^+-ATPase and Na^+-H^+ exchange in ischaemia (Tani & Neely, 1989; Kitakaze *et al.*, 1988) which may facilitate Ca^{2+} entry into myocytes. Studies using cardiac myocytes loaded with Na^+- and Ca^{2+}-sensitive dyes have shown that $[Na^+]_i$ rises as well as $[Ca^{2+}]_i$ during hypoxia (Haigney *et al.*, 1992). Removing extracellular Na^+ markedly reduced the rise in $[Ca^{2+}]_i$, suggesting that the Na^+-Ca^{2+} exchange mechanism is of importance in the development of elevated cytosolic Ca^{2+}. Some of the protective effect of the antioxidant, dimethylthiourea, in ischaemic myocyte injury is thought to be due to its ability to inhibit Na^+-Ca^{2+} exchange, rather than its action against free radicals (Ziegelstein *et al.*, 1992). The relative importance of disturbances in extracellular, sarcolemmal and mitochondrial calcium (Miyata *et al.*, 1992) causing myocyte damage is not yet fully established.

As I was developing methods of isolating ventricular myocytes from ischaemic-reperfused hearts I found that to achieve stunning, Langendorff hearts had to be exposed to physiological $Ca^{2+}{}_o$ immediately after the ischaemic episode (see sections 8.2).

Mechanism of myocardial stunning caused by oxygen free radicals
(see also 1.6.2.)

All cells within the heart, including myocytes (Turner *et al.*, 1991) are potential sources of free radicals, since all cells generate these as part of normal metabolism. Impaired ATP production in ischaemia reduces a cell's ability to handle its own radicals. Abnormalities of mitochondrial oxidative phosphorylation generate extra radicals, which cannot be cleared by the cell's usual mechanisms. Xanthine oxidase catalyses free radical production in some species, although its contribution in man is disputed. In animal models, xanthine oxidase inhibitors reduce stunning in some studies (Bolli, 1992). There is evidence from clinical studies of free radical activity during myocardial ischaemia (Bell *et al.*, 1990; Oldroyd *et al.*, 1990; McMurray *et al.*, 1992; Davies *et al.*, 1990).

It is likely that neutrophils contribute substantially to free radical production in stunned myocardium. These cells are a potent source of oxygen free radicals,

which together with hypochlorous acid and proteolytic enzymes, act in concert at sites of inflammation in the acute inflammatory response.

The exact mechanism by which oxygen free radicals modify myocardial contractility is not known. Oxygen radicals cause damage by denaturing cellular proteins and enzymes (Davies, 1987). They also oxidise polyunsaturated fatty acids (Thompson & Hess, 1986) and alter membrane permeability. Lipid peroxidation is an attractive theory but experimental evidence is presently lacking. Another possibility is damage to the sarcoplasmic reticulum (SR). Calcium uptake by myocardium is impaired in myocardial stunning (Thompson & Hess, 1986). In isolated SR preparations, exposure to oxygen free radicals depresses calcium uptake, and this can be reversed by free radical scavengers (Bolli, 1992). The sarcolemma may also be a target for free radical injury. As mentioned above, there is impairment of Na^+-Ca^{2+} exchange, Na^+-K^+-ATPase and Na^+-H^+ exchange in ischaemia (Tani & Neely, 1989) (Kitakaze *et al.*, 1988) which may facilitate Ca^{2+} entry into myocytes.

Original hypothesis: stunning is caused by nitric oxide production within cardiac myocytes

There is much experimental data, yet the mechanism of myocardial stunning is not known. Even the phenomenon itself is disputed by some. Having access to isolated myocytes I wished to address one hypothesis to explain stunning: constitutive nitric oxide synthase exists in ventricular myocytes, yet its function is not understood. My studies with L-NAME in normal myocytes showed no effect of this inhibitor on contractility, and this is confirmed by unpublished observations of Shah and Lewis. I wanted to examine the effect of substrates and inhibitors of nitric oxide synthase on myocytes from ischaemic-reperfused hearts, to determine whether constitutive nitric oxide synthase is activated in this situation.

8.1.3. **Methods**

The experimental models I tried are described in section 2.6.. The protocol for the present study is shown in Figure 35. The important modification was to introduce a period of reperfusion with Ca^{2+}-containing buffer before beginning the low calcium perfusion and enzymatic digests. A regular rhythm was usually achieved

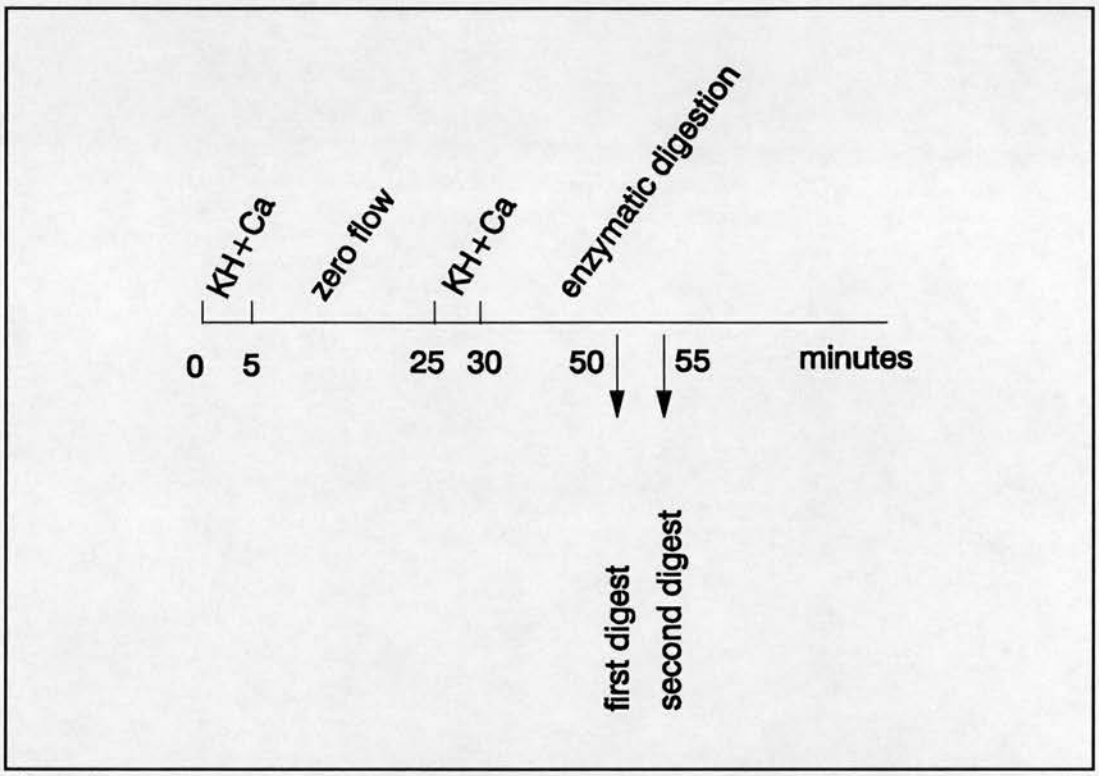


Figure 35 Protocol for production of ischaemia-reperfused cardiac ventricular myocytes from interventricular septal tissue. The heart was maintained at 35°C in a water jacket.

during reperfusion, although atrial fibrillation sometimes occurred. To ensure that only ischaemic cells were obtained epicardial tissue was discarded and the interventricular septum dissected out and used as the source of myocytes, which were then isolated in the usual way. Cells from the first digest were used to keep the time from ischaemic injury as brief as possible. It was intended to then study the effects of drugs affecting nitric oxide synthase on myocyte contractility over the following 60 min.

The protocol meant that a minimum of 25 min elapsed from the end of the ischaemic period until the first digest of cells was ready for study. *In vivo* studies

suggest that 20 min ischaemia impairs myocardial contractility for at least 1 h afterwards. Studies of the effects of 10 min hypoxia on single myocytes showed abnormalities of time to peak contraction and relaxation time which persisted for up to 50 min (Silverman *et al.*, 1991). Thus, this ought to leave a time window of about half an hour after the cells were ready in which to study their recovery from ischaemia.

Only one myocyte from each preparation could be studied by a single observer, so on some days a technician performed a concurrent study on another videomicroscopy apparatus.

The yield of myocytes was surprisingly good, often better than the yield from non ischaemic hearts. All the control and hypoxia studies used the same Langendorff apparatus.

8.1.4. **Results**

The behaviour of myocytes from ischaemic-reperfused interventricular septa followed over 60 min without pharmacological intervention is variable. The data is shown in Table 11: there are some cells where contractility is reduced at the start and subsequently improves; and there are cells where contractility never improves but fades rapidly over the time course of the experiment. The results are shown graphically in Figure 36. The solid line in Figure 36 indicates control data of mean$\pm$SEM contraction amplitude of six normal myocytes without pharmacological intervention, also followed over 60 min.

The myocytes in the table show a variable pattern of contraction. It is impossible to select at the start of the experiment which cell is stunned and recovering and which is not going to recover. Thus pharmacological studies with these cells are not helpful. This was disappointing since the myocytes appeared histologically normal.

Some early experiments with L-arginine and L-NAME superfused over cells prepared in this way were tried before analysis of all the control cells was performed. The findings with L-arginine and L-NAME were not consistent and this was

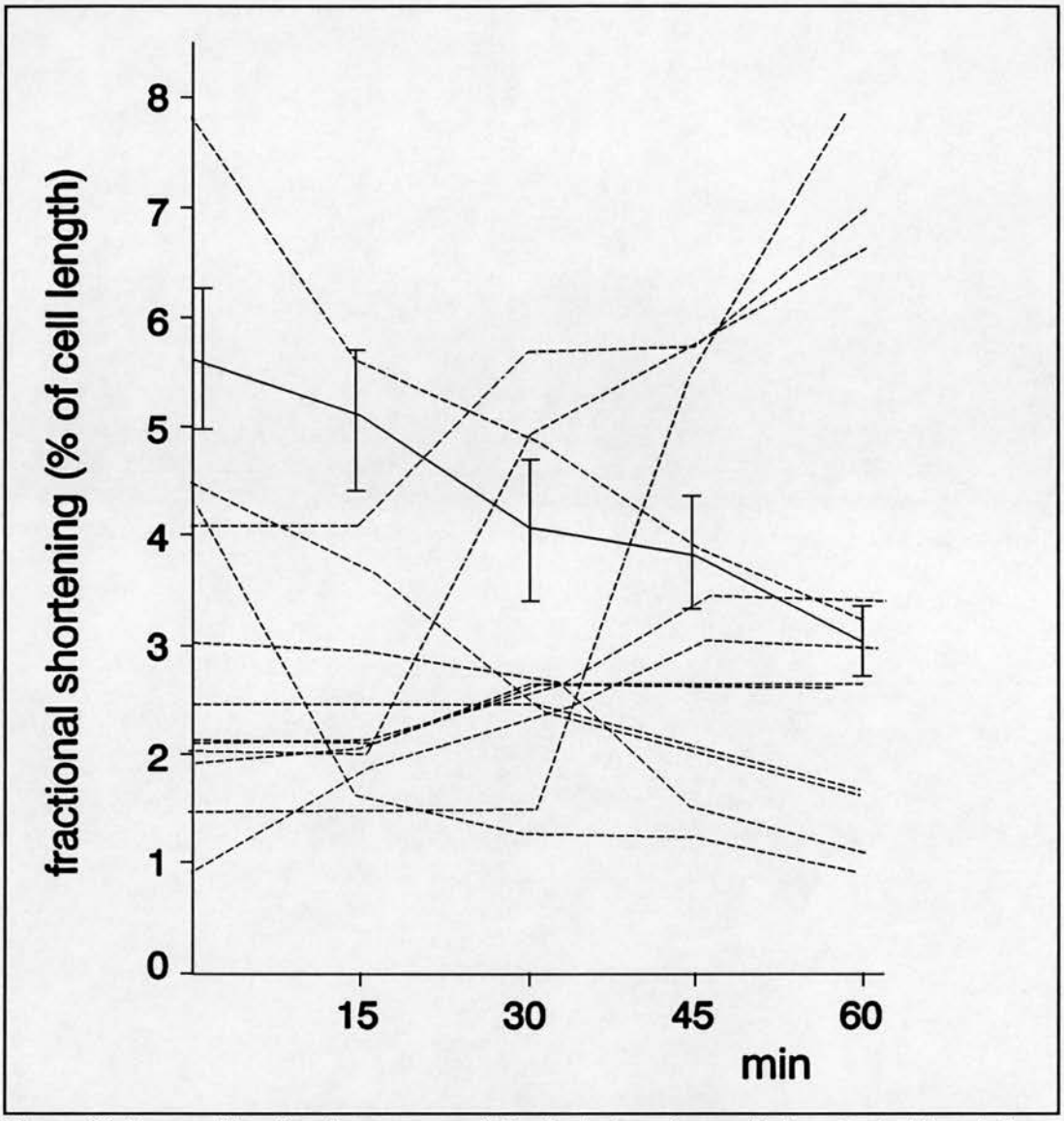


Figure 36 Contractility of cardiac myocytes following ischaemia-reperfusion. Dashed lines indicate contraction of ischaemic-reperfused cells; solid line indicates mean ± SEM of six normal myocytes.

explained subsequently by the studies of the natural behaviour of ischaemic-reperfused cells.

8.1.5. **Discussion**

A study yielding definitive information at a cellular level on mechanisms involved in post-ischaemic myocardial stunning would be worthy indeed. Unfortunately the model in the present study did not generate cells with reliable characteristics, so the hypothesis that nitric oxide production accounted for impaired

Contraction of isolated interventricular septal myocytes prepared after 20 min ischaemia during the Langendorff retroperfusion period.

	0	15	30	45	60
cell 1	4.1	4.1	5.7	5.7	7.0
cell 2	0.9	1.8	2.3	3.0	3.0
cell 3	1.9	2.1	2.6	2.6	2.6
cell 4	2.1	2.1	2.5	3.4	3.4
cell 5	2.0	2.0	5.0	5.8	6.7
cell 6	1.4	1.4	1.4	5.6	8.3
cell 7	2.4	2.4	2.4	2.1	1.7
cell 8	3.0	3.0	2.7	1.5	1.1
cell 9	4.5	3.7	2.5	2.0	1.6
cell 10	4.2	1.7	1.3	1.3	0.9
cell 11	7.8	5.6	5.0	3.9	3.3

Table 11 Fractional shortening of individual cardiac myocytes, expressed as a percentage of resting length.

contractility could not be tested. However, the results obtained examining the natural history of myocytes from ischaemic-reperfused hearts merit some explanation.

Cells were obtained from the interventricular septa of hearts subjected to zero flow for 20 min. During the period of zero flow hearts continued to beat for about 12-15 min. They were not paced, but clearly had been subjected to a substantial insult. On reperfusion a regular rhythm usually developed, although on inspection atrial arrhythmias were sometimes present. Oxygenation during zero flow from room air was excluded because only septal cells were used.

It is interesting that two types of injured cells were generated by the ischaemia-reperfusion protocol: cells which were terminally injured and cells which were recovering. In a small control study myocytes from normal hearts showed a gradual reduction in contraction amplitude over 1 h (solid line in Figure 36). A possible explanation of the behaviour of myocytes from ischaemic hearts might be that there is heterogeneity in the ability of myocytes to withstand ischaemic injury.

Alternatively, production of toxic free radicals may vary depending on the local architecture and proportion of myocytes to interstitial cells. Production of protective substances like adenosine may similarly be variable at a local level. With myocytes from normal hearts the investigator chooses the best cell available, with a stable contractile pattern within defined limits (see Chapter 2). It was not possible to impose the same limits in the present study since many of the cells behaved abnormally to start with. A better method would be to study a population of cells, similar to the method described for the neutrophil-myocyte studies in Chapter 10, and examine recovery measured simply as diastolic length. The disadvantage is that measurements of contractile function cannot be performed.

Another interesting, and unexplained, point was the high yield of myocytes despite the ischaemic episode. Perhaps the hypoxia selects out survivor cells and many injured cells die during the enzymatic digests. Without the hypoxic period these cells might have survived until the digests were ready.

Probably the main problem with the method was the time lost performing the necessary enzymatic digests. This immediate phase following onset of reperfusion is likely the key time when most of the reversible abnormalities due to ischaemia take place, but this time was unavoidably spent preparing the cells. It is during this early phase that abnormalities of sodium and calcium exchange occur (Tani & Neely, 1989; Kitakaze *et al.*, 1988), free radical production within myocyte mitochondria is likely at its peak and the sarcoplasmic reticulum is functioning least well. Altering calcium flux during the first few minutes of reperfusion after ischaemia reduces myocardial stunning (du Toit & Opie, 1992). In isolated, blood perfused rat hearts after 20 min ischaemia, reperfusion with plasma and neutrophils generates free radicals for 10 min into the reperfusion period (Shandelya *et al.*, 1993). Reperfusion with either component or plain buffer generated a short burst of free radical activity lasting 1-2 min only. This is further indication that the important events in reperfusion occur very early and these events were lost in the preparation of isolated cells in my system. I intend to address this in the future by developing a model where healthy cells from normal hearts are subjected to hypoxia while under videomicroscopic scrutiny. This is a substantial task since achieving adequately low levels of O_2 in the cell bath will involve design of new equipment. Silverman's group in Baltimore

achieve this by superfusion with argon gas (Silverman *et al.*, 1991) but few others have achieved reliable systems to study cell function under hypoxic conditions.

CHAPTER 9: NITRIC OXIDE AND CARDIAC MYOCYTE CALCIUM TRANSIENTS

9.1.1. **Introduction**

The experiments described in chapters 4-7 show that nitric oxide - from a variety of sources including: sodium nitroprusside; endothelium; a direct solution of nitric oxide itself; and, in endotoxic shock, myocytes themselves - attenuates cardiac myocyte contraction amplitude. This mechanism appears to be mediated by production of cGMP within the myocytes. The mechanism by which cGMP reduces contractility in cardiac myocytes is not yet defined, but two possibilities might explain this finding:

(i) The peak free calcium concentration (Ca^{2+} transient) within the cell during contraction is reduced by mechanisms involving either release of calcium from sarcoplasmic reticulum stores or entry of calcium from the exterior.

(ii) The sensitivity of myocyte contractile myofilaments to calcium is reduced, possibly by changes in intracellular pH, or protein phosphorylation regulated by cyclic nucleotide concentration.

9.1.2. **Excitation-contraction (EC) coupling and cardiac myocyte contraction**

This large area of research is presented in outline only, for the purposes of discussion in this chapter.

During the cardiac action potential Ca^{2+} enters myocytes via sarcolemmal Ca^{2+} channels and there may also be entry via Na^{+}/Ca^{2+} exchange across the cytoplasmic membrane. This Ca^{2+} which enters the myocyte may directly bind to myofilaments. Ca^{2+} entry from outside the cell may also be involved in the release of Ca^{2+} from the sarcoplasmic reticulum (SR), termed Ca^{2+}-induced Ca^{2+} release. Calcium ions from both the intracellular SR store and from the exterior activate myofilaments to bring about cell contraction (Bers, 1991a).

During contraction peak intracellular free calcium concentration ($[Ca^{2+}]_i$) reaches approximately 1 μM. At rest $[Ca^{2+}]$ within the cytoplasm is approximately

100 nM. Restoration of Ca^{2+} to resting levels is achieved by active extrusion of Ca^{2+} out of the cytoplasm either into the intracellular calcium store, the SR, or out of the cell either by the sarcolemmal Ca^{2+} pump or by Na^{+}/Ca^{2+} exchange (Bers, 1991a).

9.1.3. **Mechanism of ß-adrenergic inotropic effect**

Force of contraction, or inotropy, is influenced by multiple complex reactions within myocytes, and by other factors including temperature, sarcomere (and cell) length, hypoxia and pH. In general, contractility is modified either by factors which influence the amount of calcium present in the cytoplasm during the action potential, or factors which change the sensitivity of the myofilaments to Ca^{2+}. As an illustration ß-adrenergic agonists exert multiple effects on contractility (Bers, 1991b). They increase Ca^{2+} current (I_{Ca}) through the sarcolemma; they enhance SR uptake of Ca^{2+}; and they *decrease* myofilament sensitivity to Ca^{2+}. These mechanisms are mediated in part by production of cAMP within myocytes. Cyclic AMP-dependent protein kinases are activated and these cause phosphorylation of a number of cellular proteins: sarcolemmal Ca^{2+} channels; phospholamban; and troponin I. Phosphorylation of sarcolemmal Ca^{2+} channels increases Ca^{2+} entry into myocytes. ß-adrenergic agonists can also increase I_{Ca} directly by opening sarcolemmal Ca^{2+} channels by a different mechanism independent of cAMP, which involves direct binding of the ß-receptor to a G protein. Phosphorylation of phospholamban stimulates the SR Ca^{2+} ATPase, the enzyme which pumps Ca^{2+} into the SR. This promotes quicker restoration of resting $[Ca^{2+}]_i$, as well as increasing the amount of calcium stored in the SR. Conversely, phosphorylation of troponin I decreases the sensitivity of myofilaments to Ca^{2+}. The net result is that the stimulated I_{Ca} and uptake of Ca^{2+} by the SR results in a larger and faster Ca^{2+} transient.

Other agents can elevate cAMP levels in cardiac myocytes (Bers1991b). Histamine activates adenylate cyclase in myocytes and exerts a positive inotropic effect. Phosphodiesterase inhibitors, which inhibit metabolism of cAMP, also augment contractility by elevation of cAMP.

9.1.4. **Effect of acetylcholine on cAMP in cardiac myocytes**

Acetylcholine acting via muscarinic receptors inhibits adenylate cyclase, probably acting via an inhibitory G protein (Fischmeister & Hartzell, 1986). Acetylcholine also stimulates guanylate cyclase, elevating cGMP in guinea pig (Watanabe & Besch, 1975) and frog myocardium (Flitney & Singh, 1981). Cyclic GMP may stimulate cyclic nucleotide phosphodiesterase to metabolise cAMP, a second mechanism whereby acetylcholine reduces contractility. The relevance of this mechanism to the action of nitric oxide is discussed later in this chapter. Atrial natriuretic peptide (ANP) also inhibits adenylate cyclase by a G protein mechanism, and stimulates particulate guanylate cyclase, both of which stimulate cAMP breakdown in frog cardiac tissue (Gisbert & Fischmeister, 1988). This effect is seen mainly when I_{Ca} has been elevated by isoprenaline, and is less evident at basal I_{Ca}.

9.1.5. **Calcium sensitivity of the myofilaments**

Reducing sensitivity of the myofilaments to calcium reduces contractile force for the same amount of activating calcium. ß-adrenergic agonists reduce myofilament sensitivity to calcium, although their net effect is one of increasing the calcium transient. Cooling reduces myofilament sensitivity to calcium in guinea pig, rat and frog ventricle (Harrison & Bers, 1990). Shortening of sarcomere length also reduces Ca^{2+} sensitivity, as does acidosis (Fabiato & Fabiato, 1978). This may be important in ischaemia when intracellular pH falls. Increasing $[Mg^{2+}]_i$ also decreases Ca^{2+} myofilament sensitivity. Some inotropic agents, for example isomazole and pimobendan, act by increasing myofilament sensitivity to Ca^{2+} (Bers 1991a). Caffeine and theophylline also have this capability.

Phosphorylation of myosin light chains occurs in cardiac muscle as well as smooth muscle cells. This phosphorylation appears to have dual effects on Ca^{2+} sensitivity in different models, and the physiology of this reaction is not determined (Bers, 1991a). Other myofilament sites can be phosphorylated, with alteration in the sensitivity of myofilaments to Ca^{2+}. Furthermore, there are other intricate regulatory mechanisms controlling the Ca^{2+}-mediated Ca^{2+} release from SR, which affect the magnitude of the Ca^{2+} transient (Bers, 1991b). The control of myofilaments by Ca^{2+} is thus complex indeed, and the subject of intensive study.

9.1.6. **Hypothesis**

My hypothesis for this series of experiments was that nitric oxide modulates cardiac myocyte contractility by reducing peak Ca^{2+} concentration during contraction. To study this I examined the effect of nitric oxide on free [Ca^{2+}], recorded during the contraction of single cells, using the fluorescent Ca^{2+} indicator, Indo-1.

9.2.1. **Methods**

I performed these experiments under the supervision of KT MacLeod, in his laboratory at the National Heart and Lung Institute.

Isolated adult guinea pig cardiac ventricular myocytes were prepared as described in Chapter 2. Cells from the third digest were used frequently, as they appeared to have greater stability in Indo-1 labelling experiments. Briefly, 2 ml of myocyte suspension in low calcium solution (see Methods, Chapter 2) was incubated with the acetoxymethyl ester of Indo-1 (Indo-1 AM, Molecular Probes, Eugene, Oregon, USA; final concentration 5-10 μM). Myocytes were incubated for 30 min at room temperature.

Indo-1 AM, the lipid soluble ester form of Indo-1, readily enters myocytes and diffuses throughout the cytoplasm. Cytoplasmic esterases cleave the lipid soluble esters leaving the free Indo-1 acid which is membrane impermeable (Grynkiewicz, *et al,* 1985). Compartmentalisation of this indicator is known to occur but experiments in the host laboratory showed that accumulation of Indo-1 in intracellular compartments is negligible. When cells loaded with Indo-1 are "skinned," that is, the sarcolemmal membrane is removed, the fluorescence signal declines smoothly to zero (background levels) over a short time course (7 min). If Indo-1 was concentrated in compartments its decline would not follow a simple exponential function, nor would it decline to background fluorescent values.

Indo-1 loaded cells were placed in a cell bath and superfused with 2 mM Tyrode's solution at 32°C, as before. Cells were stimulated to contract at 0.5 Hz using field stimulation. Light from a 150 W xenon arc was used for epifluorescence. An interference filter was used to select the wavelength 360 nm ($\pm$10 nm bandwidth) to excite the Indo-1. After excitation, the fluorescent emitted light was split using a

dichroic mirror and the emissions at 405 and 485 nm were recorded. As $[Ca^{2+}]_i$ rises during the cardiac action potential fluorescence at 405 nm increases, and fluorescence at 485 nm decreases. The change in fluorescence at both wavelengths is expressed as a ratio, measured in arbitrary units. When measuring the effect of nitric oxide on the Ca^{2+} transient 8-10 Ca^{2+} transients from each cell were averaged.

Basal recordings of the Ca^{2+} transient were made during superfusion with control solution, and then myocytes were superfused with 10^{-6}M nitric oxide solution, as described in Chapters 2 and 5. Briefly, 2 mM Tyrode's solution was bubbled with N_2 for 4 h to exclude O_2. Nitric oxide gas was injected using a Hamilton gas syringe to make a 10^{-6}M solution. The concentration was checked with chemiluminescence. Nitric oxide solution was kept anoxic until it reached the cell bath. Myocytes were exposed for 4 min to nitric oxide or control solution, at which time recordings of Ca^{2+} transients were performed. Whether the Ca^{2+} transient returned to its previous control level was also noted.

9.3.1. **Results**

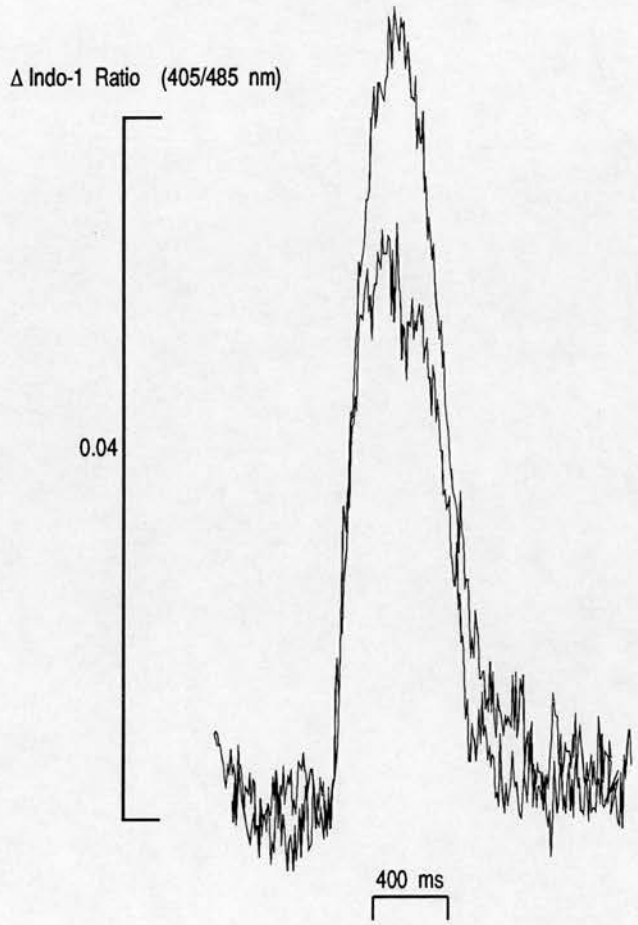
The experiments described in Chapter 5 showed that administration of 10^{-6}M nitric oxide solution attenuated cardiac myocyte contraction amplitude. In the present study nitric oxide solution reduced the calcium transient of ventricular myocytes.

Figure 37 shows a representative Ca^{2+} transient of a single cell. The larger transient shows the changes in $[Ca^{2+}]_i$ during a single cardiac action potential while the cell is being superfused with control solution. In the presence of 10^{-6}M nitric oxide the Ca^{2+} transient is reduced by approximately 30% in this cell (producing the smaller change in $[Ca^{2+}]_i$).

Nine myocytes were studied. 8-10 Ca^{2+} transients were signal averaged for each cell under control conditions, then during superfusion with nitric oxide solution. The mean data from this study are inset into Figure 37. Exposure of myocytes to nitric oxide caused a reduction in peak calcium transient of $24 \pm 7\%$ ($n=9$, $p<0.01$).

On returning to control solution the Ca^{2+} transients did not always make a complete recovery to control levels. Ca^{2+} transients did not return to their control diastolic level in six of the nine myocytes studied.

NITRIC OXIDE AND CARDIAC MYOCYTE CALCIUM TRANSIENTS



MEAN DATA

10^{-6}M Nitric oxide

Calcium transient ↓ by 24±7%

n=9 myocytes

p<0.01

Figure 37

9.4.1. **Discussion**

Nitric oxide at 10^{-6}M attenuates cardiac myocyte contraction amplitude by approximately 25%, as shown by the results of the experiments in Chapter 5. In the present study nitric oxide reduced cardiac myocyte Ca^{2+} transients by a similar amount. This suggests that in this model nitric oxide, acting on guanylate cyclase within myocytes to elevate cGMP, causes a reduction in the amount of free Ca^{2+} in the cytoplasm during the cardiac action potential. There is less calcium available for excitation-contraction coupling and contractility is decreased. These results do not rule out an additional effect on Ca^{2+} sensitivity of myofilaments, but the reduction in $[Ca^{2+}]_i$ during contraction may be adequate to account for the reduced contractility seen in contraction amplitude experiments. It is interesting, although probably coincidental, that the reduction in contraction amplitude, and the reduction in Ca^{2+} transient, are of similar magnitude. The relationship between $[Ca^{2+}]_i$ and contractile force is sigmoidal, not linear (Bers, 1991b). The relationship between $[Ca^{2+}]_i$ and cell shortening is not established.

How nitric oxide causes the reduction in Ca^{2+} transient is not explained by this study. The likely possibilities are either that Ca^{2+} entry from the exterior, or Ca^{2+} release from SR stores, is reduced by some means. A clue to a likely site of action can be drawn from the results of experiments studying ANP.

ANP acts on particulate guanylate cyclase to elevate intracellular cGMP. Cyclic GMP stimulates cyclic nucleotide phosphodiesterase which metabolises cAMP. In frog cardiac cells, using whole-cell patch-clamp techniques, ANP has been shown to reduce I_{Ca} (Gisbert & Fischmeister, 1986). ANP also activates a G protein which inhibits adenylate cyclase, and thus there are two mechanisms to reduce cAMP, which may explain the action of ANP on myocyte I_{Ca}.

As mentioned in 9.1.4., acetylcholine reduces cAMP in myocytes, both by inhibiting adenylate cyclase via a G protein, and by stimulating production of cGMP. Thus, neurohumoral pathways exist for cGMP regulation of myocyte contraction. That nitric oxide may act by a similar mechanism, elevation of cGMP, to reduce contractility, is logical.

Shah and colleagues have recently presented preliminary data on a similar

study, the influence of cGMP on Ca^{2+} transients in rat ventricular myocytes (Shah *et al*, 1993). The stable analogue of cGMP, 8-bromo-cyclic GMP (8-br-cGMP) reduced contractility but had no effect on the Ca^{2+} transient. Their conclusion was that cGMP was modifying the sensitivity of myofilaments to calcium. Their findings are thus the converse of my results. However, there are two important differences between the two experimental models. Firstly, elevation of $[Ca^{2+}]_i$ during the cardiac action potential in rat cardiac myocytes results mainly from Ca^{2+} release from SR stores. In guinea pig (and human) ventricular myocytes a greater proportion of the rise in $[Ca^{2+}]_i$ is due to calcium entry via sarcolemmal channels. Calcium handling during excitation-contraction coupling is therefore different in the two species tested. Mechanisms involving cGMP may therefore also be different.

Secondly, I performed my experiments with cells superfused with 2 mM Ca^{2+} Tyrode's solution. Shah's study was performed with 1 mM Ca^{2+} solution. If nitric oxide is modifying calcium entry via sarcolemmal channels, the effect of blocking these may be greater when external Ca^{2+} is higher.

In the present study myocyte Ca^{2+} transients did not reach baseline levels after exposure to nitric oxide solution in six of the nine myocytes studied. Recovery of contraction amplitude also appeared to be impaired, although this was not formally measured in this study. The failure of Ca^{2+} transients to return to basal values may be due to the exposure of cells to a high concentration of nitric oxide, which may be toxic. In the experiments in Chapter 5 cells were exposed to nitric oxide for 7 min. Contraction amplitude was reduced but returned to approximately 90% of normal once the control solution was reinstated. In the present study the design of the cell bath and nitric oxide delivery apparatus was different, and it is possible that the local concentration of nitric oxide was higher in these studies.

Alternatively, this may be a pharmacological, rather than a toxic, effect. Shah also found that recovery of contractility in rat myocytes was impaired after exposure to high levels of 8-br-cGMP (AM Shah, personal communication). It may be that high intracellular concentrations of cGMP have a prolonged effect on the contractile apparatus. In my early experiments using sodium nitroprusside, and myocytes in coculture with endothelium, myocyte contraction amplitude returned to baseline in most cases after exposure to the nitric oxide source. This suggests that the

conversion of sodium nitroprusside to nitric oxide within myocytes may be limited. It is also likely that the amount of nitric oxide released from endothelium in coculture which reaches myocytes causes less elevation of cGMP than administration of high doses of 8-br-cGMP.

In conclusion, administration of nitric oxide solution to cardiac myocytes reduces Ca^{2+} transient by approximately 25%. This may explain the reduction in myocyte contraction amplitude caused by nitric oxide from the sources described in earlier chapters. The results from the present study suggest that the effect of nitric oxide may be either to modulate Ca^{2+} entry into the myocytes, or modulate Ca^{2+} release from SR stores, at least in this model. Work in progress by others suggests that, in rat cardiac myocytes, nitric oxide acts on the myofilaments to modify their sensitivity to calcium. In the future, I plan to perform studies to examine the effect of 8-br-cGMP and sodium nitroprusside on guinea pig myocyte Ca^{2+} transients to resolve these differences.

CHAPTER 10: EFFECT OF NEUTROPHILS ON CARDIAC MYOCYTE CONTRACTION

Preface

When I wrote the Fellowship application for this research period there was nothing published on cellular interactions between neutrophils and cardiac myocytes. Many studies had shown the importance of neutrophils in myocardial ischaemia, and historically their role in wound repair after infarction was established. Most of the recent studies examined alterations in infarct size as a consequence of interference with neutrophil function before, during or after a period of ischaemia-reperfusion, mainly using *in vivo* dog models. But by their nature none of these studies addressed the mechanism of neutrophil-mediated myocardial injury. Whether neutrophils damage myocardium because of microvascular obstruction and resulting local ischaemia could not be excluded. Studies at a cellular level to define whether direct neutrophil-mediated myocyte injury occurred were required. These I planned to carry out.

My hypothesis was that adhesion of neutrophils to myocytes injured by ischaemia would modify contractility, and ultimately lead to myocyte death. Difficulties were identified with the different models tried. These methods, together with some promising results, are presented below.

I had some early experience of working with neutrophils, while at the Hammersmith Hospital. I examined the relative contributions of flow (shear stress) versus activation in the adherence of neutrophils to endothelial cells in a novel dynamic model. This work is included in this chapter for completeness.

INTRODUCTION

Neutrophils have the potential to damage myocardium by their release of toxic products. Whether such toxins are liberated in a random fashion by activated neutrophils within an area of injured myocardium, or whether they are targeted towards injured myocytes in particular has not yet been determined. However,

emerging evidence suggests that injured cardiac myocytes may signal their own destruction by activating such mechanisms at their cell surface. This is reviewed in depth in chapter 1, and summarised here.

10.1.1. **Neutrophil-endothelial adhesion**

At sites of inflammation neutrophils roll along endothelium by engaging and disengaging to the endothelial cells by adhesive interactions involving the neutrophil glycoprotein L-selectin (Lawrence & Springer, 1991). Tethering of neutrophils at sites of inflammation occurs via the expression of the endothelial glycoprotein P-selectin which appears on cell surfaces within minutes of endothelial activation by inflammatory mediators (Geng *et al.*, 1990). P-selectin probably mediates early neutrophil adhesion to activated or injured endothelium, since it is expressed from intracellular or cell membrane stores without the need for synthesis of new proteins (Geng *et al.*, 1990). P-selectin interacts with its carbohydrate ligand CD15 on the neutrophil (Larsen *et al.*, 1990). Neutrophils undergo an early change in their adhesion molecule presentation, with L-selectin appearing briefly first, followed by the CD18 adhesion glycoprotein complex (Vedder & Harlan, 1988; Nourshargh *et al.*, 1989).

Migration of neutrophils across the endothelial layer follows adhesion, and requires contact between the neutrophil integrin CD18 complex and the endothelial adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) (Smith *et al.*, 1988). Activated endothelium synthesises and expresses the adhesion glycoproteins, ICAM-1, and also E-selectin (formerly ELAM-1). ICAM-1 is present constitutively on endothelium at low levels but its expression is increased substantially by inflammatory mediators (Colditz & Movat, 1984). Monoclonal antibodies to CD18 reduce neutrophil infiltration into ischaemic-reperfused myocardium (Williams *et al.*, 1990) and have been shown to reduce infarct size (Seewaldt-Becker *et al.*, 1990). In the latter study a monoclonal antibody against ICAM-1 was also protective, but not all authors have found that the anti-CD18 antibody reduces infarct size, although neutrophil infiltration is decreased (Tanaka *et al.*, 1993).

E-selectin on endothelium requires *de novo* protein synthesis (Bevilacqua *et*

al., 1989). It binds to a carbohydrate on the neutrophils surface, sialylated Lewis x tetrasaccharide (Lowe *et al.*, 1990). Both E-selectin and ICAM-1 mediate neutrophil adhesion to and migration through activated endothelium.

10.1.2. **Neutrophil-cardiac myocyte interactions**

Once neutrophils have reached the extravascular space they can directly attack cardiac myocytes. Whether this damage is directed specifically towards previously injured myocytes is not known, and this was one of the main aims of my planned studies. Cardiac myocytes as well as endothelial cells are now known to express ICAM-1 in response to activation by inflammatory mediators (Smith *et al.*, 1991), and bind to neutrophil CD18 glycoprotein (Entman *et al.*, 1990). MRNA for ICAM-1 can be induced in both endothelial cells and cardiac myocytes by post-ischaemic cardiac lymph (Youker *et al.*, 1992), and recent data suggest that direct neutrophil-mediated injury of cardiac myocytes requires adhesion by the CD18/ICAM-1 mechanism (Entman *et al.*, 1991). However, expression of ICAM-1 by myocytes requires *de novo* protein synthesis (Entman *et al.*, 1990). Since it has been shown that complement activation products are present on the surface of myocytes following ischaemia, a possible mechanism of early neutrophil attachment is via a CD11b/CD18 (complement receptor 3) interaction with iC3b.

Hypotheses

I wished to study the direct interaction between activated neutrophils and cardiac myocytes. Planned early studies were:

(i) To determine whether activated neutrophils would attack healthy cardiac myocytes, myocytes primed with cytokines, or whether they would adhere specifically to injured cells.

(ii) Whether adhesion of neutrophils was required for toxicity, or whether by releasing toxic products into the medium, nearby healthy myocytes would be damaged.

(iii) Whether contractility of myocytes was affected by adhesion or presence of neutrophils.

(iv) More elaborate plans included injuring a population of isolated myocytes with hypoxia, colour labelling them and then mixing them with healthy myocytes, adding neutrophils and watching which myocytes were attacked. Adherence with ^{111}In-labelled neutrophils to quantify adhesion was also considered.

10.1.3. **Methods**

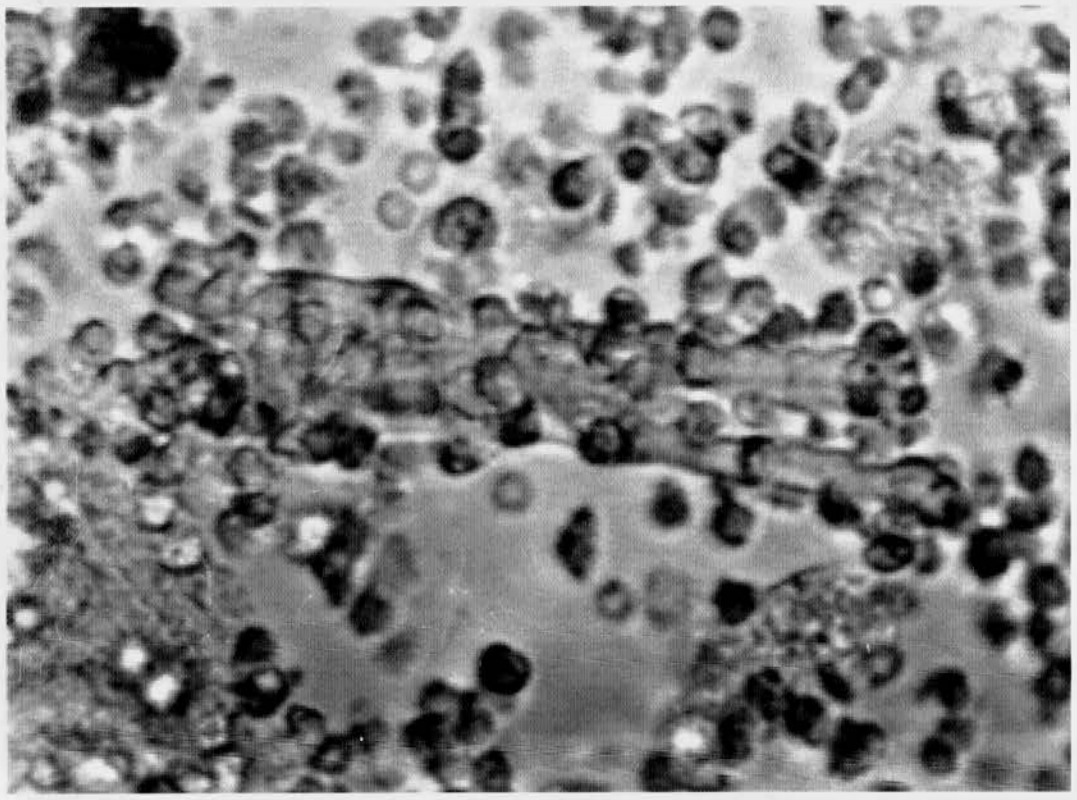


Figure 38 High power view of guinea pig ventricular myocyte with adherent neutrophils. Note irregular outline of neutrophils indicating their activated state, x400.

I had ready access to isolated guinea pig ventricular myocytes. The problem to avoid species heterogeneity was to obtain guinea pig neutrophils. A method of preparing neutrophils from guinea pig whole blood had never been well defined in the literature and exploratory trials were not fruitful. For a short while I used rabbit

blood neutrophils, since the purification method was established in the department, and prepared rabbit ventricular myocytes in the same manner as for guinea pig hearts. There were difficulties in doing both myocyte and neutrophil preparations in different laboratories concurrently so I returned to guinea pig ventricular myocytes.

Different early models of cellular interaction were explored. In the first experiments I used multiwell plates with different proportions of neutrophils and myocytes, and added recombinant human IL-1 to activate myocytes and/or the neutrophil activating oligopeptide, FMLP to activate neutrophils, in different combinations. Myocyte function could not be studied, and there were technical difficulties focusing on the multiwell plate, so I changed to using the cell bath employed in regular experiments on isolated cardiac myocytes (see chapter 2).

I performed a series of exploratory experiments using neutrophils isolated from rabbit blood together with guinea pig myocytes. Although different species were used, encouraging results were obtained.

Viewed under high power magnification addition of neutrophils activated with FMLP after 30 min exposure to IL-1 2U/ml had a marked effect on contractility of a healthy myocyte. Neutrophils adhered in large numbers to the myocyte, as illustrated in Figure 38. That the neutrophils were not merely proximal but were adherent to the myocyte was shown by their adhesion in the presence of flow. With adhesion of neutrophils contraction became sluggish and the myocyte fibrillated before finally rounding up in irreversible contracture. Such experiments were carried out at room temperature in the absence of flow, except when flow was turned on to test adherence. In control studies without neutrophils myocytes continued to contract for >1 h under these conditions, so the effects of neutrophils were not a result of stasis, nor of changes in temperature.

I modified this model so that under lower magnification between five and nine myocytes were viewed simultaneously. Instead of measuring fractional shortening only diastolic length was measured, since shortening cannot be determined accurately at low power. Control studies showed that healthy myocytes only gradually reduced their diastolic length over 1 h. I therefore examined the effects of addition of neutrophils, with the addition of FMLP in some cases to a field of healthy myocytes. Cells which rounded because of irreversible injury were readily visible and could be

recorded as terminally injured cells. $0.5x10^6$ neutrophils were added to a field of about 100 myocytes already adherent to the coverslip in the 200 μl cell bath and were allowed to settle for 5 min. Flow was then turned on for a few minutes to wash away excess neutrophils and then the experiments was followed over 1 h in the absence of flow.

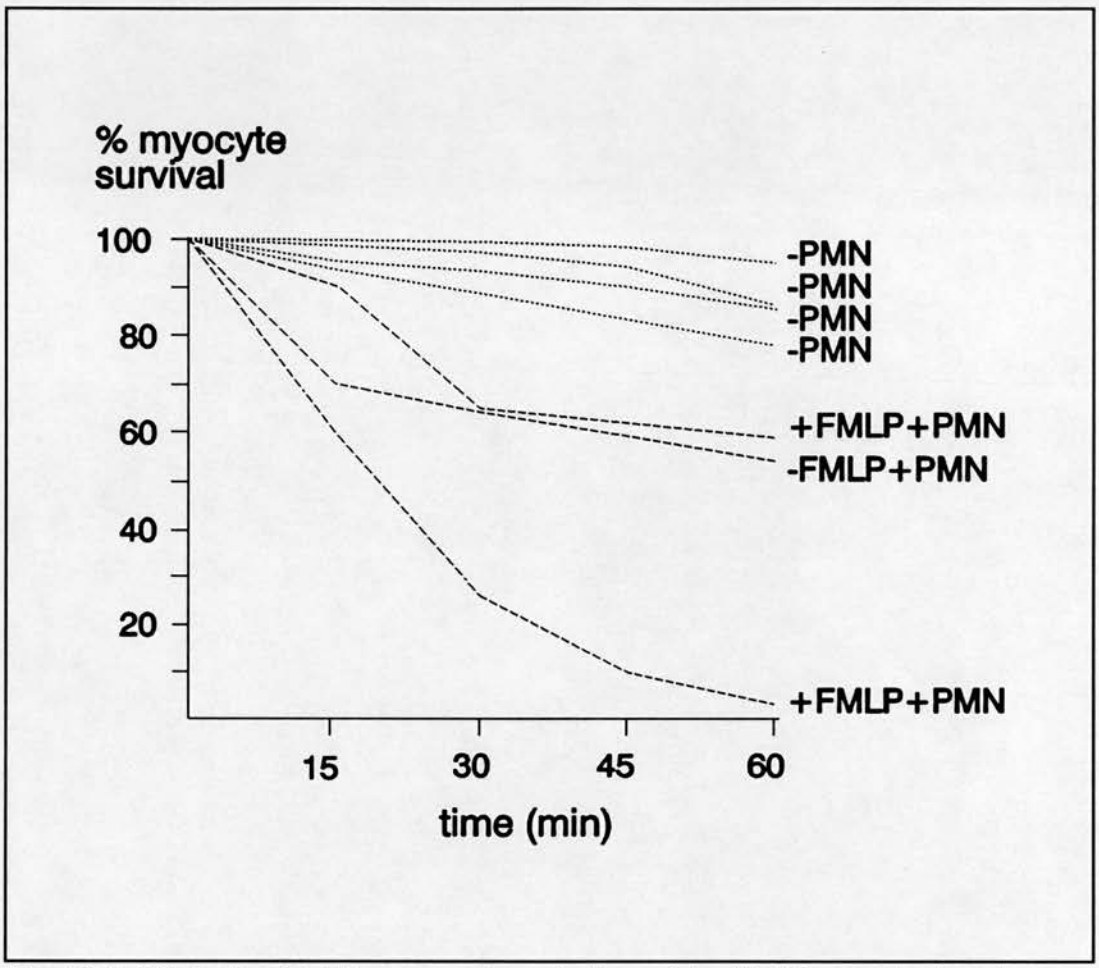


Figure 39 Effect of neutrophils on myocyte survival over 1 h. N=5-9 myocytes in each group. +PMN, neutrophils added; FMLP, formylated Met-Leu-Phe peptide.

10.1.4. **Results**

Figure 39 shows the effect on myocyte contractility, shown as survival of isolated myocytes after addition of neutrophils. In the absence of neutrophils myocyte survival was nearly 100% over 1 h. Addition of neutrophils activated fully with FMLP rapidly injured previously healthy myocytes in one set of myocytes, although

in another set only a proportion of myocytes were injured. In another set addition of neutrophils without FMLP also directly injured some of the myocytes.

10.1.5. **Discussion**

This exploratory study showed that addition of neutrophils directly damages cardiac myocytes. Activation with FMLP tended to increase the cytotoxicity but even the trace amounts of endotoxin present in the system seemed to be enough to prime the neutrophils to an oxidant state. Activation of the myocytes by cytokines was not required for adhesion in this model. Although the study was performed without flow, observational experiments showed that once neutrophils were adherent to myocytes, they were not dislodged at even high flow rates. This is persuasive evidence for a direct adhesion between myocyte and neutrophil.

These studies provide data which addressed in part my hypotheses. Activated neutrophils did appear to adhere to apparently healthy myocytes, as well as to rounded cells. Myocyte priming with cytokines was not required. This is in contrast to the results from Smith's group in Houston (Smith *et al.*, 1991; Youker *et al.*, 1992; Entman *et al.*, 1990). In their studies incubation of cardiac myocytes with IL-1, TNFα or IL-6 was necessary to induce ICAM-1 expression, which was a prerequisite for neutrophil-myocyte adhesion. They also found that adhesion was dependent on neutrophil activation with inflammatory mediators. Antibodies to either CD18 or ICAM-1 substantially inhibited neutrophil-myocyte adhesion.

However, their studies do not explain fully what happens *in vivo*. Neutrophils accumulate in the heart following ischaemic damage shortly after reperfusion begins (see section 1.6.1.). Yet in their *in vitro* experiments 4 h are required to induce new protein in myocytes for neutrophil adhesion. There must be another, earlier signal for neutrophil attraction and adhesion to damaged myocardium. Very recently, a surface adhesion molecule with properties similar to PAF has been found to be expressed early in post-ischaemic cardiac myocytes (CW Smith, personal communication). Its structure and physiological significance have not been defined but it is tempting to suggest a role for this molecule in early ischaemia-reperfusion injury, since PAF antagonists appear to moderate post-ischaemic myocardial injury

(see section 1.6.4.).

The present study shows that contractility of myocytes is markedly modified by addition of neutrophils, measured as loss of diastolic length. Although it was evident that fractional shortening was profoundly altered before irreversible cell injury, the edge detection system usually used to measure contractility could not be used because of the presence of large numbers of neutrophils which made definition of myocyte extremities difficult. A way round this would be to use only a few neutrophils, and this could then be extended to investigate what ratio of neutrophils to myocytes was required for cell killing. Anderson and Entman have found that adhesion of two or three neutrophils is enough to cause myocyte oxidant injury (DC Anderson, personal communication, and (Entman *et al.*, 1992)). Whether this is the same for modification of contractility is not known. In my studies I wished to establish first of all whether neutrophils directly injured myocytes. Future experiments could then address specific issues to dissect this mechanism.

While these early studies provided interesting information on neutrophil-myocyte interactions, to perform a satisfactory programme of experiments would require a restructuring of laboratory practice to work in an endotoxin free environment. It is likely that trace amounts of endotoxin activates myocytes, much as it activates other immunocompetent cell types (CW Smith, personal communication). Studies of cardiac myocyte immunological behaviour need comparisons between activated and unactivated states, otherwise parameters cannot be defined.

10.1.6. **Conclusions and future perspectives**

In conclusion, laboratory evidence is accumulating that myocardial injury during reperfusion after ischaemia is a real phenomenon. Neutrophils exert potent effects on myocyte contractility and survival, and inhibition of neutrophil function or adhesion to myocytes improves recovery from ischaemia in animal models. Studies in patients have yet to confirm these findings. In the large trials of thrombolysis, an excess of deaths on the first day following reperfusion could be due to injury caused by reperfusion. But these trials were not designed to address this phenomenon so

overinterpretation of data is dangerous (Fox, 1992). Proper randomised trials of agents to block neutrophil function are required.

While some possible therapeutic agents, such as monoclonal antibodies to neutrophil adhesion molecules, are available, the size and cost of a carefully conducted, randomised trial would be substantial. Thrombolysis in myocardial infarction is so successful, and the mortality in the large trials so low (10-11%, ISIS-3 collaborative group, 1992) that additional benefit over current strategies would require the study of many thousands of patients to test the ultimate efficacy and safety of such a therapy. When the cost of producing such agents in large quantities becomes economic, such studies may be performed.

Encouragingly however, new low molecular weight carbohydrate agents which bind to the oligosaccharide moieties of adhesion receptors are becoming available. These block neutrophil-cardiac myocyte adhesion and are cheaper to produce, easier to manufacture and are potentially more readily administered to patients. Such agents are nearing clinical trials (K Mullane, personal communication). Another potential agent to reduce inflammation in myocardial injury is the soluble CR1 receptor, already shown to be of benefit in animal studies (Weisman *et al.*, 1990). Prostacyclin analogues which switch off neutrophil function is another area of therapeutic interest (Simpson *et al.*, 1987a), although hypotension is a problem in patients (Blauth *et al.*, 1987). In the near future results of trials using drugs which act either as analogues of adenosine or stimulants of its release are expected, and these and other early modulations of the inflammatory response may offer important gains in the management of myocardial ischaemia.

10.2. NEUTROPHIL ENDOTHELIAL INTERACTIONS

10.2.1. **Introduction**

The mechanism of neutrophil adhesion to endothelium has been described in chapter 1 sections 1.6.8.-1.6.9., and in the introduction to this chapter. Since adherence of a moving neutrophil to stationary endothelium involves loss of motion in a continuously flowing medium I set out to examine the relative contributions of flow of perfusing solution and level of neutrophil activation to adhesion to endothelial cells. My hypothesis was that adhesion of neutrophils to endothelium might be directly related to the rate at which perfusing solution was flowing.

10.2.2. **Methods**

Bovine pulmonary artery endothelial cells were prepared without enzymatic digestion, in the same manner as bovine aortic endothelium, described in chapter 2. The cells were cultured on to 150 μm diameter Cytodex beads and 0.5 ml placed in a cell column made from the barrel of a 2 ml syringe, as shown in Figure 40. The system was maintained at 37°C with a water jacket round the cell column.

Circulating human neutrophils were prepared from volunteer donor blood under the direction of Dr CH Haslett, using sedimentation through a discontinuous carbohydrate gradient, similar to the preparation of rabbit neutrophils described in chapter 2. Isolated neutrophils were labelled with ^{111}Indium in tropolonate, and washed three times in Hank's buffered salt solution (Ca^{2+} 1 mM). Since partial activation of neutrophils is difficult to achieve, cells were either used in unactivated form, or were fully stimulated with 10 min incubation in lipopolysaccharide 100 ng/ml, followed by 3 min exposure to FMLP 10^{-6}M. The level of neutrophil activation was determined by shape change assay. Quiescent neutrophils are round; activated cells have an irregular outline, readily distinguishable as irreversible activation.

70-80x10^6 neutrophils in a final volume of 3 ml were gently loaded into superfusing solution running at 0.5 ml/min through the endothelial cell column. Flow

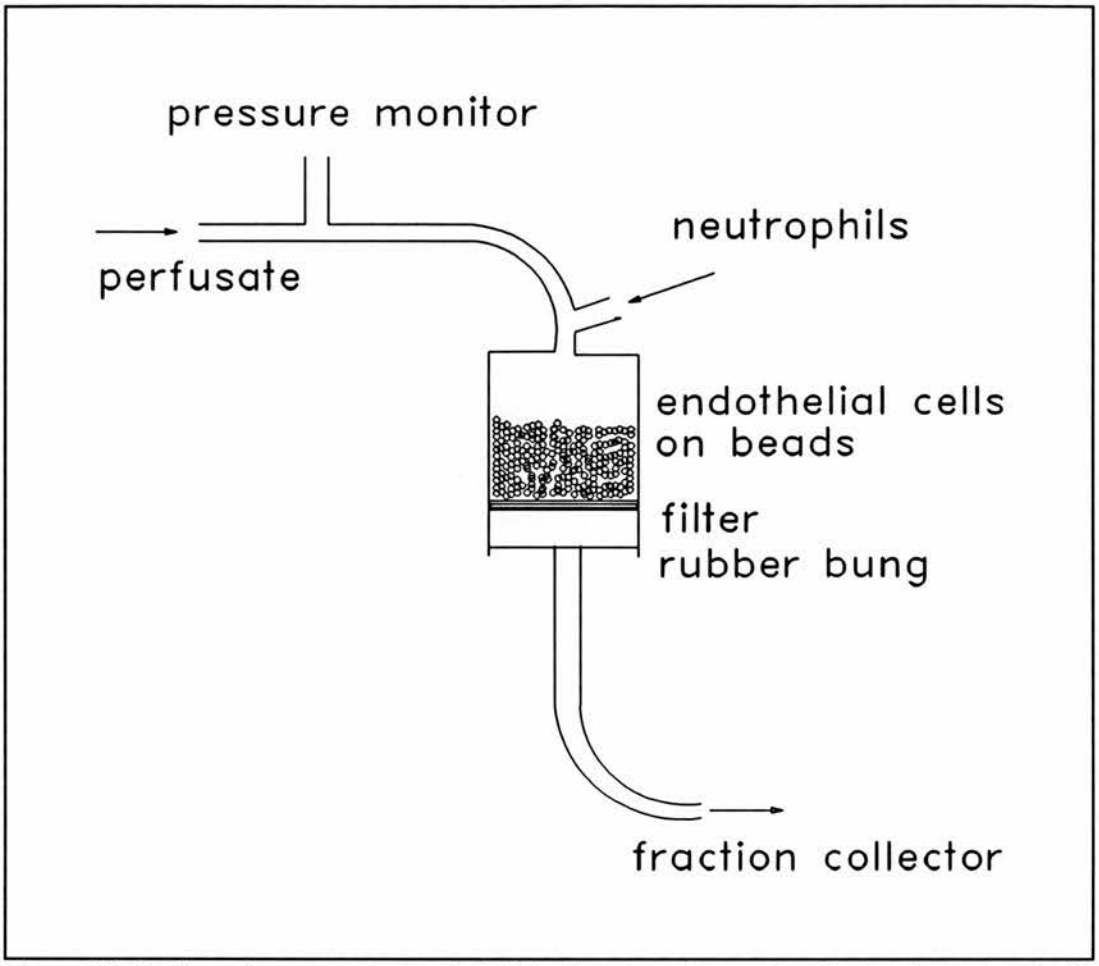


Figure 40 Bovine pulmonary artery endothelial cells cultured on to Cytodex beads, perfused with human neutrophils.

was maintained at 0.5 ml/min for 16 min, then increased to 1 ml/min for 16 min, then to 2 ml/min for 16 min. 1 minute collections were made and counted for ^{111}In activity using a gamma counter. Counts at each flow rate were totalled and residual ^{111}In activity on the endothelial column, *i.e.* the proportion of neutrophils retained, was counted. Neutrophils adherent to the filter were counted but were a small proportion of the total.

Pressure in the system was measured on one occasion through a side arm using a Grass pressure transducer. The recordings are shown below:

0.5 ml/min : 10 mm Hg

1 ml/min : 32 mm Hg

2 ml/min : 63 mm Hg

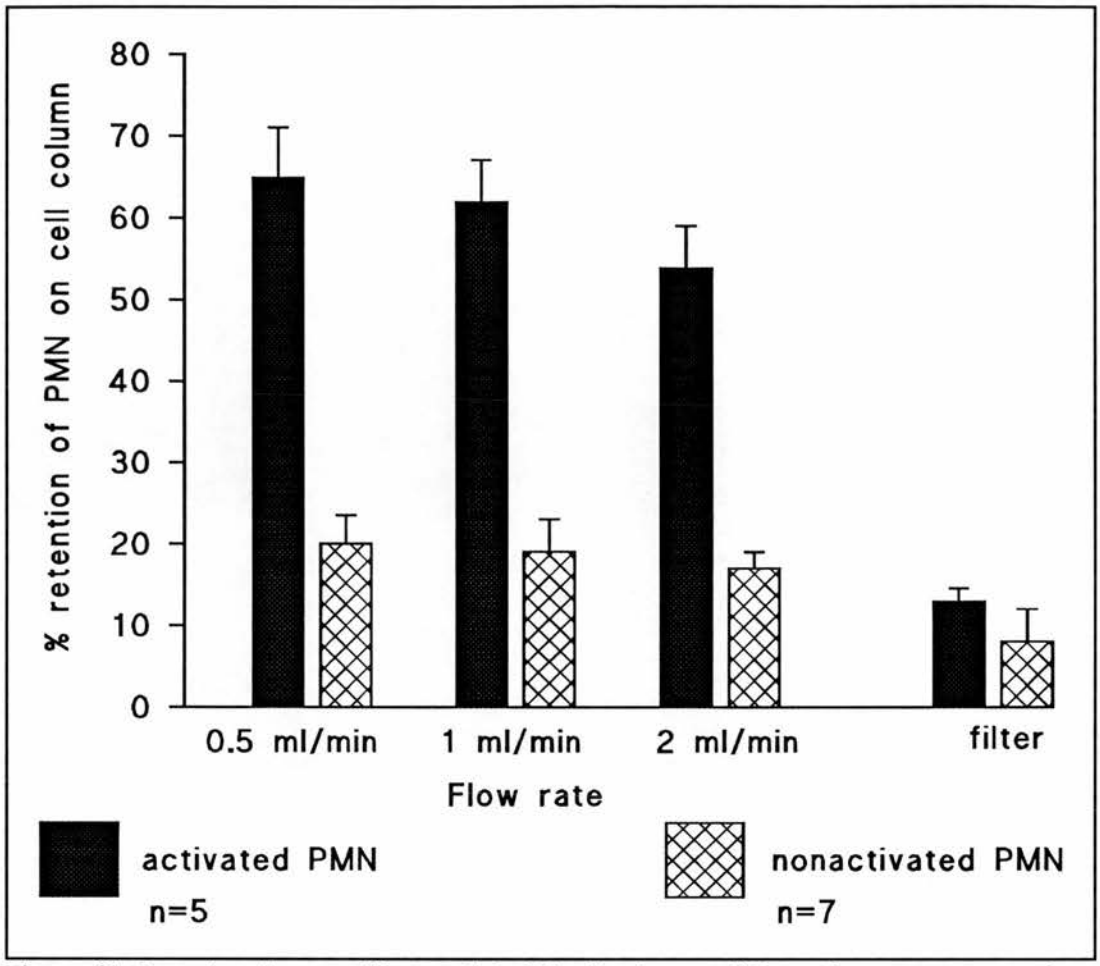


Figure 41 Retention of neutrophils on endothelial cell column at different flow rates, expressed are the percentage of total injected neutrophils. PMN, polymorphonuclear leukocytes (neutrophils).

10.2.3. **Results**

Figure 41 shows the adhesion of neutrophils to the endothelial column at different flow rates. It is clear that adhesion, measured as retention of neutrophils on the endothelial column, is related to the level of activation, and not to the rate of perfusing flow.

N=5 experiments with activated neutrophils; activation recorded as >90% shape changed. N=7 experiments with nonactivated neutrophils; shape change <20%.

10.2.4. **Discussion**

My hypothesis was that adhesion of neutrophils to endothelium was related to the rate at which perfusing solution flowed past the adherent neutrophils. In fact neutrophil activation had a greater influence on adhesion than did a fourfold increase in flow. A possible explanation of this is that the strength of neutrophil binding to endothelium is enough to resist the increased shear force generated by the perfusing fluid. Endothelium is now known to rapidly express the adhesion glycoprotein P-selectin within minutes of endothelial activation by inflammatory mediators (Geng *et al.*, 1990). P-selectin may mediate early neutrophil adhesion to activated or injured endothelium, since it is expressed from intracellular or cell membrane stores without the need for synthesis of new proteins. P-selectin interacts with its carbohydrate ligand CD15 on the neutrophil (Larsen *et al.*, 1990). The way to prove this would have been to examine the effect of blocking antibodies to either the endothelial or neutrophil adhesion molecule, but this was not possible.

An alternative explanation is that neutrophils aggregate in the spaces between endothelial cell beads and some of the apparent adhesion to the column is in fact due to neutrophil-neutrophil adhesion. I tried to address this by making sections of glutaraldehyde-fixed columns and having transmission electron microscopy performed. Unfortunately the cytodex beads fragmented on the apparatus used to cut sections, both at room temperature and after freezing with liquid nitrogen, so this was not pursued. Thus this study does not answer fully the question it was designed to test, but it does provide interesting data on a dynamic model of neutrophil-endothelial adhesion, an area under study by other research groups.

One implication of this study is that at sites of inflammation, recruitment of neutrophils from nearby patent vessels can occur down chemotactic gradients while blood continues to flow. This is important because neutrophils need to adhere to endothelium with a bond strong enough to resist the shear force of flowing blood. That they do so in the present system where the pressure within the microcirculation is as high as 63 mm Hg and flow 2 ml/min suggests that the adhesion is strong indeed. Estimation of the haemodynamics in my experimental model is difficult because there will be convectional accelerations round the curved surfaces of the

beads. Nevertheless, assumptions can be made to estimate the size of the lumen of the cell column.

The approximate size of the channels between beads in an assumed cross section of the system is shown below:

area of endothelial cell column	$= \pi r^2$
	$= \pi \times 0.5^2 = 0.79\ cm^2$
area of a cell bead	$= 2 \times 10^{-5}\ cm^2$
area of a square of side equal to the diameter of a bead	$= 2.5 \times 10^{-5}\ cm^2$
$area_{square}$ - $area_{bead}$ = area of perfusing channel	$= 5 \times 10^{-6}\ cm^2$
No. of squares covering area of column	$= area_{column}/area_{square} = 31600$
therefore, effective area of perfusing channel	$= 31600 \times 5 \times 10^{-6}\ cm^2 = 0.158\ cm^2$

At 1 ml/min flow in the experimental model is roughly equivalent to laminar flow in a vessel 0.158 cm^2 in cross section, or 0.44 cm in diameter, with an intraluminal pressure of 32 mm Hg. These measurements are within physiological limits.

Thus it can be suggested from the present study that the level of activation is more important than the rate at which blood flows through vessels in determining neutrophil adhesion to endothelium. It can be suggested that neutrophils can move to extravascular sites of inflammation from nearby vessels without the need for sluggish blood flow. Flow in these vessels will be increased because of vasodilatation due to the effects of inflammatory mediators and rather than being washed away, neutrophils will stick to endothelium and migrate towards the source of chemotactic factors. When flow is reduced, the adhesive properties of neutrophils, combined with increased stiffness (Worthen *et al.*, 1989), will encourage retention and this may contribute to the no reflow phenomenon sometimes observed during reperfusion after coronary occlusion.

CHAPTER 11: GENERAL DISCUSSION

Cardiac myocytes metabolise sodium nitroprusside, but not organic nitrates, to generate nitric oxide

The importance of nitric oxide in the function of many different cell types has been recognised only recently. The main thrust of most of the work in this thesis was to establish whether nitric oxide has a role in modulation of myocardial contraction. Its influence on healthy cells was demonstrated first by the effects of sodium nitroprusside on cardiac myocyte contraction.

Nitrovasodilators act by being metabolised locally to release nitric oxide. Contractility of cardiac myocytes was not affected by the organic nitrates, glyceryl trinitrate and isosorbide dinitrate, but administration of sodium nitroprusside reduced myocyte contraction. This suggests that cardiac ventricular myocytes do not contain the enzyme(s) necessary to generate nitric oxide from organic nitrates. The effect of sodium nitroprusside was blocked by the inhibitor of soluble guanylate cyclase, methylene blue. This suggests that nitric oxide is acting by stimulating production of cGMP within the myocytes.

Nitric oxide attenuates cardiac myocyte contraction

Administration of nitric oxide solution provided direct evidence for an attenuating effect of nitric oxide on contractility. That this effect is mediated by production of cGMP within the myocytes is further supported by the experiments using 8-bromo-cGMP. This stable analogue of cGMP had an effect similar to sodium nitroprusside, reducing myocyte contraction.

The effect of elevating cGMP within myocytes appears to be to limit the rise in $[Ca^{2+}]_i$ during the cardiac action potential, as shown by the experiments described in Chapter 9. This means that either the entry of calcium from outside the cell is decreased, or the release of stored calcium from intracellular stores is reduced. How nitric oxide modifies these mechanisms occur is not known.

Nitric oxide from stimulated endothelium attenuates cardiac myocyte contraction

The work was extended into a physiological coculture model of myocytes with endothelium. Again, nitric oxide from stimulated endothelium attenuated contractility of myocytes. Inhibition of endothelial nitric oxide production with L-NAME blocked this effect. The attenuation was small, in the range 10-25%, when nitric oxide in solution, or when derived from nitrovasodilator drugs or the endothelium acted on healthy myocytes.

Cardiac myocytes from healthy animals do not demonstrate appreciable nitric oxide synthase activity in their basal state

Administration of either inhibitors of nitric oxide synthase, or its substrate L-arginine, had no effect on contraction of normal myocytes in my study. This has since been confirmed by others (Amrani *et al.*, 1992), (Shah and Lewis, personal communication). These important control studies show that quiescent myocytes do not express nitric oxide synthase in an active form. Constitutive nitric oxide synthase has now been shown to be present in normal, healthy cardiac myocytes (Schultz *et al.*, 1992; Balligand *et al.*, 1993) but does not appear to generate appreciable nitric oxide. Inhibitors and substrates of nitric oxide synthase had no effect on contractile function of cells in their basal state, either in the experiments of this thesis, or the experiments of others (Amrani *et al.*, 1992; Schultz *et al.*, 1992; Balligand *et al.*, 1993).

It has now been shown by others that nitric oxide produced endogenously within myocytes may modify the inotropic response to ß-adrenergic stimulation in cardiac myocytes (Balligand *et al.*, 1993). This phenomenon only occurred following exposure of myocytes to isoprenaline. The same paper also provided evidence that nitric oxide mediates the inhibitory effect of carbachol on myocytes.

The report by Finkel which showed nitric oxide-dependent depression of papillary muscle contraction after a few minutes' exposure to cytokines (Finkel *et al.*, 1992) does not fit with the data on isolated myocytes. Possibly a cytokine-dependent nitric oxide synthase enzyme is induced very rapidly in hamster papillary myocytes. Alternatively, the cytokines are acting on another cell type within the papillary muscle which quickly generates nitric oxide.

A role for nitric oxide in myocardial contraction?

There are important implications of this work. The coronary microcirculation is in close proximity to cardiac myocytes and endothelium-derived nitric oxide may have a tonic effect on myocardial contractility, analogous to the release of EDRF from endothelium in the vasculature. Within the circulation, local blood flow is probably regulated by tonic modulations in neurohumoral mechanisms which affect nitric oxide release, and its subsequent effect on vascular smooth muscle. Whether some form of regulation of myocyte contraction by nearby endothelium occurs within the heart is not known. Importantly, others have now shown that nitric oxide production within myocytes may be involved in the regulation of inotropic responses of myocytes to ß-agonists, and nitric oxide may mediate the effects of negatively inotropic cholinergic pathways within myocytes (Balligand *et al.*, 1993).

The circumstantial evidence from the work of this thesis, together with studies of endocardial endothelium, which shows that ventricular myocytes are sensitive to exogenous nitric oxide in physiological amounts, argues that a relationship between endothelium and cardiac myocytes may exist. This relationship may be particularly important when cardiac architecture is disturbed in myocardial disorders.

In ventricular hypertrophy and in cardiomyopathic conditions, there is accumulation of interstitial cells and fibrous tissue. The distance from vasculature to myocyte is increased, and myocyte contractility may thus be affected by altered diffusion of nitric oxide. In the whole organ this may result in abnormalities of contraction and relaxation. Although the effects of nitric oxide in my studies are mainly on contraction amplitude, papillary muscle work suggests that there are important effects on the rate of contraction and relaxation as well. Thus alterations of nitric oxide diffusion into myocytes may be important in both systolic and diastolic dysfunction.

Does nitric oxide contribute to post ischaemic myocardial stunning?

Coronary endothelial dysfunction occurs early in ischaemia-reperfusion injury and impaired production of nitric oxide may be important in this condition. It is tempting to suggest that an abnormality of nitric oxide production affects myocyte contraction as well. I was keen to test whether loss of contractility of myocytes following ischaemia was the result of activation of constitutive nitric oxide synthase. There is biochemical evidence of the presence of this enzyme in human myocardium (De Belder *et al.*, 1993) and in animal ventricular myocytes (Schulz *et al.*, 1992). As shown by the work of this thesis the function of this enzyme in health is not known (Brady *et al.*, 1992). As mentioned above, cytokines can activate nitric oxide synthase activity in hamster papillary muscle with a time course too short for induction of new protein (Finkel *et al.*, 1992). The experimental model designed in my studies to address the effects of reversible ischaemia did not function satisfactorily so the hypothesis that activity of constitutive nitric oxide synthase contributes to postischaemic myocardial stunning remains untested.

Production of nitric oxide within cardiac myocytes contributes to their impaired contractility in endotoxic shock

Probably the most important finding of the work of this thesis, readily applicable to the clinical situation, was the study of behaviour of cardiac myocytes from animals with endotoxic shock. Myocytes from healthy animals do not have appreciable nitric oxide synthase activity. In endotoxaemia, the contractility of cardiac myocytes is markedly reduced. A substantial proportion of this reduction is due to the induction of nitric oxide synthase and subsequent production of nitric oxide within the myocytes themselves. Again, similar to the situation in many other tissues overproduction of nitric oxide accounts for important functional abnormality. Endotoxic shock is associated with myocardial depression, exacerbating the profound haemodynamic changes which occur in this condition. Nitric oxide production within cardiac myocytes may contribute to the contractile impairment in endotoxic heart failure.

A randomised trial of the effect of L-arginine analogues in patients with endotoxic shock is nearing completion (P Vallance, personal communication). This

study examines haemodynamic profiles but does not measure myocardial contractility directly. Vallance and I plan to carry out a second clinical study with echocardiography as well as haemodynamic measurements, to address this question.

Recently, inducible nitric oxide synthase activity in biopsies of human myocardium from patients with dilated cardiomyopathy has been demonstrated (De Belder *et al.*, 1993). The key question from that study is: does production of nitric oxide within myocytes in dilated cardiomyopathy account for impaired contractility? Endomyocardial biopsy yields many cell types, including endocardial endothelium, vascular endothelium, vascular smooth muscle and fibroblasts, all of which show inducible nitric oxide synthase activity in inflammatory conditions (Brady, 1993). Thus their methodology cannot answer the central question, but the implication is that a common mechanism, overproduction of nitric oxide within myocardium, may cause myocardial depression in both acute and chronic inflammatory conditions.

The evidence that nitric oxide production from inducible nitric oxide synthase activity may impair myocardial contractility suggests exciting possibilities for specific therapy in cardiomyopathic conditions in the future.

APPENDIX: PUBLISHED WORK RELATING TO THIS THESIS

1. **Brady AJB**, Warren JB, Poole-Wilson PA, Williams TJ, Harding SE.
Nitric oxide attenuates cardiac myocyte contraction.
American Journal of Physiology 1993;265:H176-H182..

2. **Brady AJB**, Poole-Wilson PA, Harding SE, Warren JB.
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3. **Brady AJB**, Poole-Wilson PA.
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British Heart Journal 1993;70:103-105.

4. **Brady AJB**, Williams FM, Williams TJ.
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5. Warren JB, **Brady AJB,** Taylor GW.
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ABSTRACTS

8. **Brady AJB**, JB Warren, SE Harding, PA Poole-Wilson.
Effect of nitric oxide in aqueous solution, and 8-bromo-guanosine-3':5'-monophosphate, on contraction of isolated, guinea-pig cardiac ventricular myocytes.
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10. **Brady AJB**, Warren JB, Poole-Wilson PA, Williams TJ, Harding SE.
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12. **Brady AJB,** Warren JB, Poole-Wilson PA, Harding SE.
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13. **Brady AJB,** Meagher LC, Haslett CH, Warren JB.
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15. **Brady AJB**, RU Naqvi, JB Warren, PA Poole-Wilson, KT MacLeod.
Nitric oxide attenuates contraction amplitude and intracellular calcium transients of contracting guinea pig cardiac ventricular myocytes.
Circulation 1993; in press.

Research Awards/

16. **Brady AJB.**

Nitric oxide and cardiac myocyte contraction.

1993 British Cardiac Society Young Research Workers' Award.

British Heart Journal 1993; 69(5) Suppl: P57.

17. **Brady AJB.**

Nitric oxide and cardiac myocyte contraction.

American Heart Association Melvin L. Marcus Young Investigator Awards.

Circulation 1993; in press.

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Nitric oxide attenuates cardiac myocyte contraction

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Brady, Adrian J. B., John B. Warren, Philip A. Poole-Wilson, Timothy J. Williams, and Sian E. Harding. Nitric oxide attenuates cardiac myocyte contraction. *Am. J. Physiol.* 265 (*Heart Circ. Physiol.* 34): H176–H182, 1993. —Cardiac muscle fibers have microvessels in close proximity, the distance from the nearest capillary being no greater than 8 μm. We performed experiments on isolated, electrically stimulated, contracting guinea pig cardiac myocytes to test whether NO from endothelium or nitrovasodilators or directly superfused in solution might affect myocyte contractility. In endothelium-myocyte coculture experiments, 10^{-7} M bradykinin reduced myocyte shortening by 11 ± 3.5%. This effect was abolished in the presence of N^G-nitro-L-arginine methyl ester and was unaffected by indomethacin. Sodium nitroprusside, but not organic nitrovasodilators, reduced myocyte contraction amplitude by 23% at 3×10^{-5} M. This effect was reversed by methylene blue. Superfusion with NO solution had an effect similar to sodium nitroprusside, as did exposure to 8-bromoguanosine 3′,5′-cyclic monophosphate. Thus the present study shows that cardiac myocyte contraction is attenuated by NO, which appears to act via production of guanosine 3′,5′-cyclic monophosphate within the myocytes. Because cardiac myocytes in vivo are in such close proximity to endothelium, the effects of endothelial products on cardiac myocyte contractility may be important in myocardial function.

endothelium; nitrovasodilator; guanosine 3′,5′-cyclic monophosphate

THE CORONARY MICROCIRCULATION lies in close proximity to cardiac muscle, so that most myocytes are within 8 μm of their nearest capillary (16, 17). This short diffusing distance means that myocytes may be influenced by vasoactive factors produced by adjacent endothelium. Endothelium-derived relaxing factor (EDRF), considered now to be NO or a closely related, short-lived compound, is capable of relaxing smooth muscle of the vascular system, airways, gut, and urinary tract (14). The intracellular mechanism involves the stimulation of soluble guanylate cyclase to increase intracellular levels of guanosine 3′,5′-cyclic monophosphate (cGMP) with a subsequent reduction in intracellular calcium.

It is not known whether vasodilator factors from endothelium affect contraction of cardiac myocytes. However, we have shown recently that the contractility of isolated cardiac myocytes can be attenuated by generation of NO within the myocytes themselves (2). Myocardial contractility is markedly impaired in the later stages of endotoxic shock. We found that the contractility of cardiac myocytes from endotoxin-treated animals was substantially reduced and this could be reversed by inhibition of either NO synthase or guanylate cyclase, indicating NO synthase activity within the myocytes themselves. This phenomenon was not present in cardiac myocytes from normal animals.

The primary aim of the present study was to examine whether isolated, functioning cardiac myocytes from healthy animals are sensitive to exogenous NO. The sources of NO used were *1*) stimulated endothelium in a primary coculture model, *2*) nitrovasodilator compounds, and *3*) NO gas dissolved in solution. Because NO acts by elevating intracellular levels of cGMP, the effects of the stable analogue, 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), were also studied, as well as the effects of the inhibitor of guanylate cyclase, methylene blue.

The secondary aim of this study was to establish whether cardiac myocytes can metabolize nitrovasodilators other than sodium nitroprusside to generate NO. Although nitrovasodilators act by being metabolized to produce NO, different nitrovasodilators are metabolized by different pathways (9). It has been shown recently that porcine coronary smooth muscle cells metabolize glyceryl trinitrate (GTN) to generate NO by a plasma membrane enzyme (5). In contrast, sodium nitroprusside requires only a reduction step to generate NO, which requires NADPH, NADH, or a thiol and takes place in the microsomes (8). Although the effects of nitrovasodilators on cardiovascular hemodynamics are established in both patients and experimental models, the changes induced in coronary flow, peripheral resistance, venous filling pressure, and pulmonary capillary wedge pressure will obscure any specific action of nitrovasodilators on myocardial contractility, i.e., the extent and rate of myocardial shortening. The isolated, functioning cardiac myocyte is a suitable model to study contractility in the absence of hemodynamic changes.

MATERIALS AND METHODS

Guinea pig cardiac myocytes were isolated by enzymatic digestion, as described previously (12). A drop of myocyte suspension was placed in a 200-μl Perspex chamber on the stage of an inverted microscope, either on a 22-mm-diam plain glass cover slip or on a cover slip with confluent bovine aortic endothelium, and superfused at 2 ml/min with 2 mM calcium Krebs-Henseleit buffer [containing (in mM) 119.1 NaCl, 4.7 KCl, 0.94 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.5 glucose] bubbled with 95% O_2-5% CO_2 at 32 ± 0.5°C. Temperature was monitored by a thermistor in the cell bath. Cells were allowed to adhere for 4 min without flow and were then stimulated electrically to contract using a bipolar stimulator delivering pulses of 30 V/4 ms at 0.5 Hz.

Contractility of individual myocytes was determined as described in detail elsewhere (11). Briefly, contraction amplitude and velocity of shortening of electrically stimulated myocytes were recorded using a videomicroscopy length-detection system and analyzed by computer-signal averaging. This system had a time resolution of 20 ms and a spatial resolution of 1 in 256, which allowed typically 10–15 sampling points within a single contraction and relaxation. Six consecutive contractions were signal averaged to produce data under each particular set of conditions. Myocyte contraction amplitude was calculated as a percentage of the resting length, and time to peak contraction

and time from peak contraction to return to 90% of resting length (relaxation time) were recorded. The following criteria were defined to identify healthy, viable, isolated myocytes (12): *1*) only rod-shaped cells without sarcolemmal blebs were examined; *2*) cells that exhibited spontaneous contractions were excluded; and *3*) cells that displayed a variable baseline contraction to electrical stimulation at 2 mM calcium were rejected.

Bovine aortic endothelial cells were isolated and passaged by nonenzymatic methods (26). The cells had normal cobblestone morphology, factor VIII immunoreactivity, and high levels of angiotensin-converting enzyme activity. They were used between *passages 10* and *18* and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (GIBCO, Paisley, UK), 200,000 IU/l penicillin, 200 mg/l streptomycin, and 480 mg/l glutamine. Cells were passaged onto sterile glass cover slips contained in multiwell plates and used 2-3 days later when confluent.

In the endothelium-myocyte coculture experiments 10^{-7} M bradykinin was used to stimulate release of EDRF. The ability of the endothelium to generate NO when stimulated with bradykinin was confirmed in separate studies by chemiluminescence using a Sievers Nitric Oxide Chemiluminescence Analyzer (Denver, CO) and also by bioassay using endothelial cells grown on microcarrier beads in a superfusion cascade system (data not shown).

To allow accumulation of endothelium-derived factors, superfusing buffer flow was halted 5 min before recordings were made. An indwelling thermocouple and heating element were used to maintain bath temperature, and 95% O_2-5% CO_2 gas was superfused over the cell bath to maintain oxygenation and pH. The temperature of the system was controlled so that paired recordings were made at matched temperatures. Control studies showed that a change of +1.0°C from 32°C reduced contraction amplitude by 5 ± 0.5% (SE; $n = 8$), and similarly, a change of −1°C increased contraction by 5 ± 0.5% ($n = 7$). For the recordings made during coculture experiments, bath temperature was maintained at 32.6 ± 0.2°C. Some endothelium-myocyte studies were performed in the presence of 10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME) to inhibit production of NO or in the presence of 10^{-5} M indomethacin to block prostaglandin synthesis.

In the experiments examining the effect of nitrovasodilators on isolated myocyte contractility, sodium nitroprusside solutions were used over the concentration range 10^{-8} to 3×10^{-5} M, and containers were wrapped in foil to exclude light. Concentrations of GTN (Lipha, Middlesex, UK) and isosorbide dinitrate (ISDN; Schwarz Pharma, Chesham, Buckinghamshire, UK) were used over the dose range 10^{-6} to 3×10^{-5} M. Measurements were made on 6-10 myocytes at each dose of nitrovasodilator. Effects of each nitrovasodilator were compared randomly with control measurements recorded before or after myocyte exposure to nitrovasodilator at each concentration. Concentrations of nitrovasodilators were chosen randomly, and cumulative dose-response protocols were avoided. Myocytes were exposed to each concentration of nitrovasodilator for 10 min and were not exposed to more than one nitrovasodilator. The time course of the study allowed for measurements made on individual myocytes within 45 min of stabilization. This avoided the reduction in myocyte contraction amplitude that can occur in studies of >2 h in duration.

In further experiments 5×10^{-6} M methylene blue (David Bull Laboratories, Warwick, UK) was added as an inhibitor of guanylate cyclase. In another series of experiments the effect of the stable analogue of cGMP, 8-BrcGMP (Boehringer Mannheim, Lewes, East Sussex, UK), was examined using cumulative doses, over the range 10^{-6} to 3×10^{-5} M. All other drugs and chemicals were from Sigma (Poole, Dorset, UK) except where stated.

Some experiments were performed to examine the effect of NO dissolved in physiological solution on cardiac myocyte contraction. Aliquots (100 or 1,000 μl) of NO gas (99% pure; Merck, Poole, Dorset, UK) were injected using a Hamilton gas syringe (Reno, NV) into 300 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered 2 mM Ca^{2+}-Tyrode solution [containing (in mM) 150 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 5.0 Na-HEPES, and 10.0 glucose; pH 7.40] that had been bubbled vigorously with nitrogen gas for 4 h to remove oxygen. This yielded stock solutions of $\sim 10^{-6}$ to 10^{-5} M dissolved NO (for 100 and 1,000 μl, respectively), and the stock solutions were kept anoxic. Aliquots were withdrawn and superfused into the cell bath for 7 min. NO solution was protected from exposure to air until it reached the cell under study. The NO concentration of the 10^{-6} M NO solutions was confirmed using the NO chemiluminescence analyzer to be within the range $1\text{-}3 \times 10^{-6}$ M NO on multiple samplings of different 100-μl NO solutions. The 1,000-μl NO solutions were assumed to contain 10-fold more NO. Control experiments were performed using the same Tyrode solution, bubbled with 100% nitrogen.

Values are expressed as means ± SE except where stated. Statistical differences between baseline and stimulated endothelium- or nitrovasodilator-influenced contraction were tested using Student's two-tailed *t* test for paired data or one-way analysis of variance (ANOVA). The dose dependence of nitrovasodilator effects was assessed using regression analysis.

RESULTS

Figure 1 shows a representative signal-averaged trace (of 6 contractions) of the effect of 10^{-5} M sodium nitroprusside on contraction of a single cardiac myocyte. In this experiment contraction amplitude was reduced from 5.8 to 4.7% of resting length, a reduction in myocyte contraction of 19%, with no effect on either time to peak contraction or relaxation time. The difference between the fractional shortening in the presence of nitrovasodilator was expressed as a percentage of the baseline contraction (Fig. 2). GTN and ISDN over the dose range

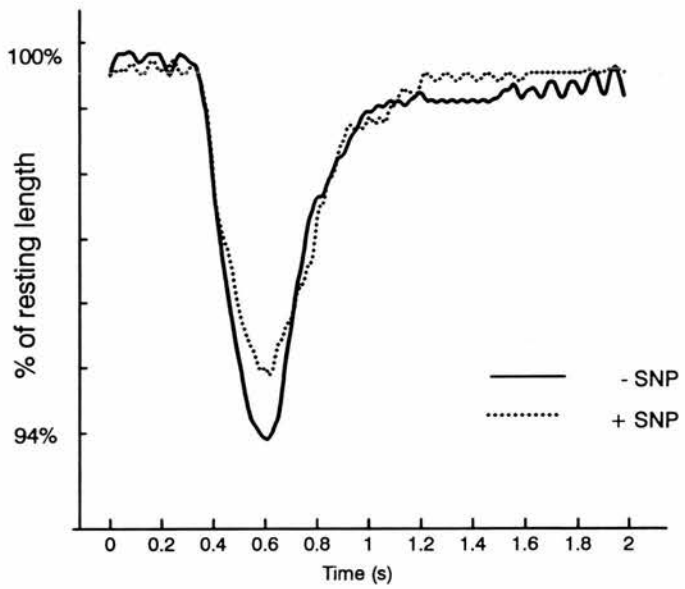

Fig. 1. Representative signal-averaged trace of 10^{-5} M sodium nitroprusside (SNP) effect on myocyte contraction. Solid line, baseline contraction; dotted line, effect of SNP.

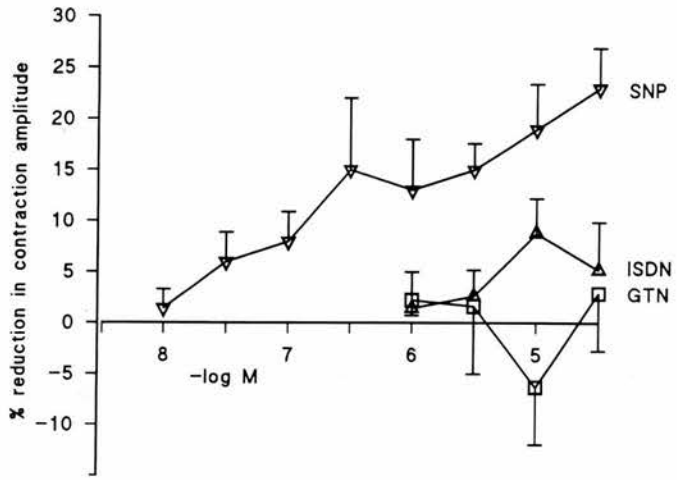

Fig. 2. Effect of different nitrovasodilators on myocyte contraction amplitude (differences expressed as %reduction of control contractions). ISDN, isosorbide dinitrate; GTN, glyceryl trinitrate. Values are means ± SE of 6–10 experiments. SNP significantly reduced contraction amplitude at concentrations $>3 \times 10^{-8}$ M. $P < 0.02$ by analysis of variance.

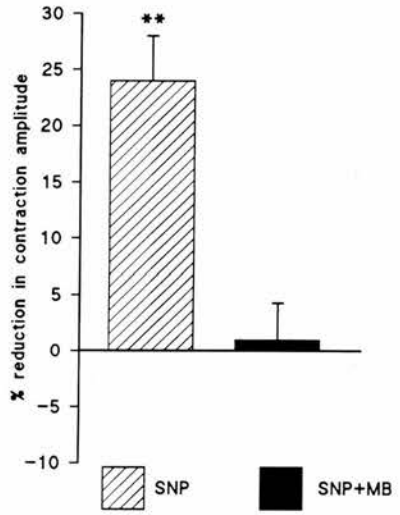

Fig. 3. Effect of 10^{-5} M SNP on myocyte contraction ± 5×10^{-6} M methylene blue (MB). Differences are expressed as %reduction of control contractions. Values are means ± SE of 6 experiments. ** $P < 0.01$, both SNP vs. control and SNP vs. SNP + MB.

10^{-6} to 3×10^{-5} M had no effect on contraction amplitude of contracting myocytes. Sodium nitroprusside reduced contraction amplitude: the reduction was significant ($P < 0.02$, ANOVA) at concentrations of 3×10^{-8} M or above. Regression analysis showed that the effect of sodium nitroprusside was concentration dependent ($P < 0.001$), although it was not possible to determine whether the curve was monophasic or biphasic. The effect of sodium nitroprusside was reversible and repeatable, suggesting that the generation of cyanide was not a cause of the reduced contraction amplitude.

In further studies 5×10^{-6} M methylene blue was added to all solutions and reversed the reduction in myocyte shortening caused by sodium nitroprusside (Fig. 3). In control studies, methylene blue itself had no effect on myocyte contractility over the same time course as these experiments. However, superfusion of methylene blue for more prolonged periods (>30–60 min) caused cells to become hypercontractile and, subsequently, to fibrillate in 10 of 12 cardiac myocytes studied.

Compared with control, none of the nitrovasodilators had a significant effect on time to peak contraction (baseline, 0.14 ± 0.01 s; nitrovasodilator, 0.14 ± 0.01 s; pooled data from all nitrovasodilator experiments, n = 12 groups of paired studies). Similarly, relaxation times were not affected by nitrovasodilators (baseline 0.27 ± 0.01 s; nitrovasodilator 0.28 ± 0.01 s; pooled data from same experiments).

To examine the effect of endothelium-derived NO on myocyte contractility, myocytes were studied in primary coculture with confluent endothelium. To determine whether unstimulated endothelium affected baseline characteristics of contraction, the contractility of myocytes on endothelium was compared with that of myocytes on glass. Contraction amplitude of myocytes adherent to quiescent endothelium (5.3 ± 0.6% of resting length; n = 12) was not different from that of myocytes adherent to glass cover slips (5.3 ± 0.3%; n = 32). There was no difference in time to peak contraction (0.10 ± 0.02 s on endothelium, n = 11; 0.12 ± 0.02 s on glass, n = 9) or relaxation time (0.30 ± 0.04 s on endothelium, n = 11; 0.30 ± 0.03 s on glass, n = 9).

In coculture experiments 10^{-7} M bradykinin was used to release NO from the endothelium. Myocyte contraction amplitude was reduced by 11 ± 3.5% ($P < 0.03$). This effect was abolished by the presence of 10^{-4} M L-NAME and was not reduced by 10^{-5} M indomethacin (Fig. 4). Although the bradykinin-induced reduction in contraction was greater in the presence of indomethacin, this further reduction was not significantly larger than that of bradykinin alone. Bradykinin-stimulated NO release had no effect on either time to peak contraction or relaxation time (Table 1).

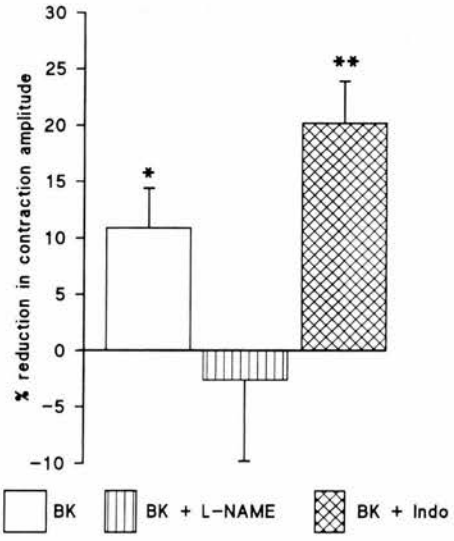

Fig. 4. Cardiac myocytes in coculture with endothelium. Change in contraction amplitude after addition of 10^{-7} M bradykinin (BK), ± 10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME), and ± 10^{-5} M indomethacin (Indo). Differences are expressed as %reduction of control contractions. Values are means ± SE of 12 (control ± BK), 11 (BK ± L-NAME), and 6 (BK ± Indo) experiments. * $P < 0.03$. ** $P < 0.01$.

Table 1. *Isolated guinea pig cardiac myocytes with cultured endothelium: effect of 10^{-7} M bradykinin-stimulated NO release on time to peak contraction and relaxation time*

	Control	Bradykinin	10^{-4} M L-NAME	10^{-4} M L-NAME + Bradykinin	10^{-5} M Indomethacin	Indomethacin + Bradykinin
Time to peak contraction, s	0.10±0.02	0.10±0.02	0.10±0.02	0.10±0.02	0.11±0.02	0.11±0.02
Relaxation time, s	0.30±0.04	0.28±0.03	0.30±0.02	0.28±0.03	0.38±0.03	0.34±0.03

Values are means ± SE of 12 [control ± bradykinin (BK)], 11 [N^G-nitro-L-arginine methyl ester (L-NAME) ± BK], and 6 (indomethacin ± BK) experiments.

In control experiments without endothelium, bradykinin had no effect on contraction amplitude (fractional shortening: control 3.7 ± 0.4% vs. 3.8 ± 0.4% in the presence of bradykinin, $n = 8$) and did not affect either time to peak contraction or relaxation time (data not shown). In further control studies on isolated myocytes L-NAME had no significant effect (+1.0 ± 3.6% increase) on contraction amplitude ($n = 9$, P = not significant).

Figure 5 shows the effect of direct superfusion with 10^{-6} M and 10^{-5} M NO solution on myocyte contraction amplitude compared with control. Nitrogen-gassed control solution had no effect on myocyte contraction; thus the effect of the NO solution was not due to hypoxia. The reduction in contractility seen with the NO solutions was similar to that caused by 3×10^{-5} M sodium nitroprusside.

The stable cGMP analogue, 8-BrcGMP, caused a concentration-related reduction in myocyte contractility, similar in magnitude to the effect of sodium nitroprusside (Fig. 6).

DISCUSSION

In the present study we have shown that the contraction of cardiac myocytes is attenuated by NO from endothelium, NO from sodium nitroprusside, or NO in aqueous solution. The effect of stimulated endothelium on myocyte contraction was blocked by the substrate inhibitor of NO synthase, L-NAME. Importantly, L-NAME had no effect on isolated myocyte contraction in control studies. In a previous study, we showed that another specific NO synthase inhibitor, N^G-monomethyl-L-arginine, did not affect contractility of healthy cardiac myocytes (1). Therefore, as in our study of myocyte behavior in endotoxic heart failure (2), it appears that cardiac myocytes themselves do not synthesize appreciable NO in their normal state and also that L-NAME has no other effects on the contractile apparatus.

Direct superfusion with a solution of dissolved NO had an effect similar to sodium nitroprusside, causing a substantial reduction in myocyte contractility. Although the amount of NO converted to nitrite on exposure to air within the cell bath is unknown, because the NO solution was protected from exposure to air until it reached the cell under study, sufficient NO from a solution of ~10^{-6} M could reach the myocytes unchanged with an effect similar to 3×10^{-5} M sodium nitroprusside. A maximal effect was seen at 10^{-6} M NO, thus cardiac myocytes are more sensitive to NO than to either sodium nitroprusside or the stable analogue of the second messenger of NO, 8-BrcGMP. A relatively high concentration of 8-BrcGMP was needed, consistent with the natural amplification of a biological signal, whereas receptor-mediated events, e.g., stimulation by NO, are amplified by the second messenger systems. Alteratively, this may reflect the difficulty of entry of 8-BrcGMP into myocytes.

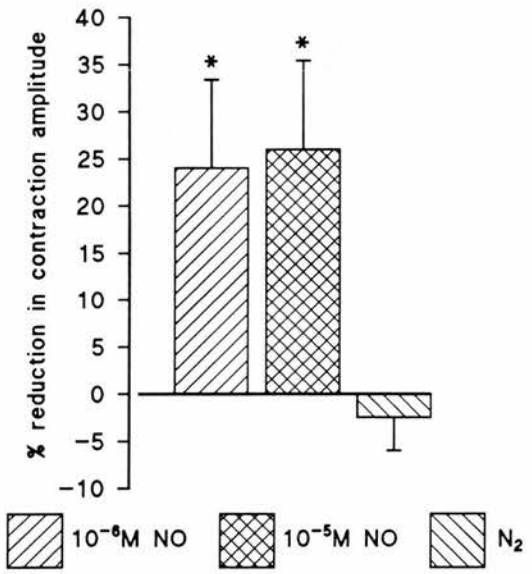

Fig. 5. Effect of 10^{-6} and 10^{-5} M NO solutions and N_2 solution on myocyte contraction amplitude. NO, NO-Tyrode solution; N_2, N_2-bubbled Tyrode. Differences are expressed as %reduction of control contractions. Values are means ± SE of 5 (10^{-6} M NO), 5 (10^{-5} M NO), and 13 (N_2) experiments. * $P < 0.05$. ** $P < 0.01$.

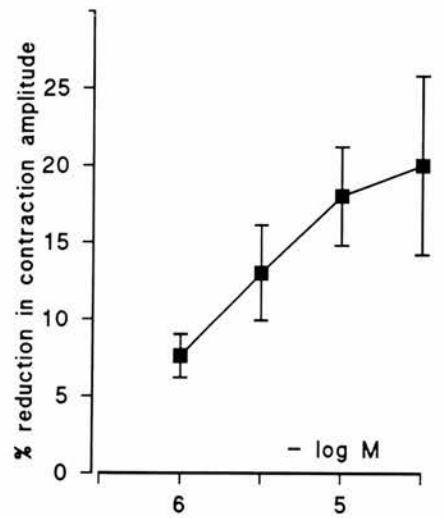

Fig. 6. Effect of 10^{-6} to 3×10^{-5} M 8-bromoguanosine 3′,5′-cyclic monophophate (8-BrcGMP) on myocyte contraction amplitude. Differences are expressed as %reduction of control contractions. Values are means ± SE of 5–7 experiments. $P < 0.025$ at each concentration of 8-BrcGMP compared with baseline contraction.

The lack of response of myocytes to either GTN or ISDN suggests that these cells, unlike vascular smooth muscle cells (5), do not possess the plasma membrane enzyme necessary for the liberation of NO from those compounds. NO solution and sodium nitroprusside had a greater effect on contraction amplitude than did stimulated endothelium, probably because the local concentration of NO generated within the myocyte from the nitroprusside, or administered directly, was higher than that made available by exogenous stimulation of endothelium.

In support of the hypothesis that important interactions exist between vascular lining cells and the myocardium, previous studies have shown that endocardial endothelium influences adjacent myocardial cells by releasing at least two humoral factors that have opposing effects on papillary muscle contraction (23). This was first described by Brutsaert and co-workers (4), who showed that damage to the endocardial surface of the papillary muscle causes a reduction of ~20% (at 2.5 mM Ca^{2+}) in maximum isometric tension produced by the muscle, brought about by an earlier onset of isometric relaxation but with no effect on the velocity of contraction. This effect occurs in endocardium-intact preparations with administration of lipid-soluble analogues of cGMP or with sodium nitroprusside and atrial natriuretic peptide (substances that increase myocardial cGMP by stimulating soluble and particulate guanylate cyclase, respectively), which reduce papillary muscle contractility in a manner similar to endocardial damage (4, 22, 23). From experiments using cultured endocardial cells on beads in a cascade system, endocardial endothelium appears to release both EDRF and an as-yet-unidentified substance that both augments contraction and prolongs relaxation (23). Restoration of endocardial products to endocardium-damaged papillary muscles both increases the force of isometric contraction and prolongs the relaxation phase after contraction in this system. The quantitative relationship between this as-yet-unknown endocardial product and endocardial EDRF is yet to be fully established, although the unidentified contraction-prolonging factor appeared to be dominant in these experiments (22).

The effect of sodium nitroprusside in papillary muscles (23) is of similar magnitude to the reduction in contraction seen in the present study. In papillary muscle experiments, small changes in time-dependent variables can be demonstrated. These changes are within 20 ms (23), and whereas our studies detected no effects on either times to peak contraction or relaxation times, this may because the time resolution of our videomicroscopy edge-detection device is itself of the order of 20 ms.

Implicit in the hypothesis for the present study is that coronary microvascular endothelium releases NO, but there is yet no direct evidence that this occurs. We used nonenzymatically cultured bovine aortic endothelium for the cardiac myocyte-endothelium coculture experiments, since it is both readily obtained and a known source of NO. But important differences exist between the endothelium lining large arteries and endothelium within the microvasculature. For example, coronary microvessels are less sensitive than the aorta to the exogenous nitrovasodilator, GTN, but relax equally to NO solution, albeit at a higher concentration (20). This implies that metabolism of organic nitrates in the coronary microvasculature is less efficient than elsewhere in the circulation, perhaps because of a relative deficiency of the enzymes required (5) or a deficiency of available sulfhydryl groups necessary for generation of NO from GTN. Importantly, NO has been shown to be a vasodilator in microvascular preparations from diverse parts of the peripheral circulation (6, 10, 15, 27). It has been demonstrated recently in isolated, perfused hearts that 5-hydroxytryptamine stimulates NO release from the coronary microvasculature, measured by coronary sinus nitrite sampling, and this is accompanied by a reduction in the time to reach peak ventricular pressure (21). These preliminary data suggest that coronary microvascular endothelium, like microvascular endothelium elsewhere, is capable of releasing factors that modulate nearby muscle contraction.

Support for a functional relationship between NO and the myocardium comes from three recent reports. Finkel et al. (7) showed that the negative inotropic effects of inflammatory cytokines on papillary muscle are mediated by generation of NO within the muscle itself. Schulz and colleagues (19) demonstrated the presence of both the constitutive and inducible forms of the NO synthase enzyme in rat cardiac myocytes, and we have shown that in experimental endotoxemia cardiac myocyte contractility is markedly impaired by a mechanism involving NO production within the myocytes themselves (2). Thus cardiac myocytes contain the biochemical apparatus necessary to both generate and handle NO. Our evidence from the present study that healthy cardiac myocytes are sensitive to exogenous NO in three different delivery systems supports the hypothesis that microvascular endothelium influences cardiac myocyte contraction by activation of NO-sensitive guanylate cyclase, although NO production by microvascular endothelium within the heart remains to be demonstrated.

The mechanism by which elevation of cGMP may cause a reduction in myocyte contraction is not well understood. In frog cardiac myocytes, cGMP inhibits the L-type inward calcium channel current (I_{Ca}) by stimulation of adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiesterase, but in rat ventricular myocytes cGMP predominantly inhibits this calcium current by a mechanism involving cGMP-dependent protein kinase, independent of any effect on cAMP levels (13, 25). Furthermore, cGMP has both stimulatory and inhibitory actions on different phosphodiesterases in rat cardiac myocytes and under certain experimental conditions has a stimulatory effect on I_{Ca} (13). Over the short time course of our experiments methylene blue, an inhibitor of guanylate cyclase, reversed the effects of NO from sodium nitroprusside. However, superfusion with methylene blue for prolonged periods >30–60 min caused the cells to contract much more strongly before fibrillating. Whether this represents an action mediated by changes in cGMP or some toxic action of methylene blue was not demonstrated in our system.

An alternative explanation of the effect of NO on cardiac myocytes is that the elasticity of proteins forming

the cytoskeleton, rather than the actin-myosin myofibrils, is modified by NO. Important changes in cytoskeletal proteins occur in ischemia (24) and in cardiomyopathy (18), but these changes probably evolve over a long time course. Whether NO can cause transient changes in cellular ultrastructure has not been described.

That NO affects cardiac myocyte contractility may have important consequences. It may be that in normal hearts, capillary endothelium produces EDRF, which has a tonic effect on contraction of nearby myocytes. With the development of cardiac hypertrophy or dilatation in disease states, the normal architecture and relationship between microvasculature and myocytes becomes disturbed with increased diffusion distance between endothelial cell and cardiac myocyte. Subsequent loss or attenuation of the tonic influence of NO on myocyte contractility may then be detrimental to myocardial function. It is of interest that sodium nitroprusside, but not the organic and more commonly used nitrates, ISDN and GTN, was metabolized to generate NO within cardiac myocytes. In vivo, this could be masked by the widespread hemodynamic changes after nitrovasodilator administration. In patients with unstable angina, sodium nitroprusside showed no difference with respect to hemodynamic changes when compared with GTN (3); it may be that vascular smooth muscle is more sensitive than cardiac muscle to the actions of sodium nitroprusside, and thus vasodilator effects are seen before changes in myocardial contractility develop.

In conclusion, our results show that the contraction of isolated cardiac myocytes is reduced by NO derived from either endothelium or sodium nitroprusside or administered directly. Because the coronary microcirculation is in such close proximity to cardiac myocytes, endothelium-derived NO may have an important effect on myocardial contractility.

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rapid communication

Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia

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Brady, Adrian J. B., Philip A. Poole-Wilson, Sian E. Harding, and John B. Warren. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. *Am. J. Physiol.* 263 (*Heart Circ. Physiol.* 32): H1963–H1966, 1992.—We investigated whether increased nitric oxide (NO) synthase activity within cardiac myocytes contributes to the depressed cardiac contractility observed in endotoxic shock. Isolated ventricular myocytes were studied to examine the effects of substrates and inhibitors of NO synthase on myocyte contractility. When stimulated electrically, the resting length of myocytes from control animals shortened by 5.3 ± 0.3% (means ± SE, n = 32). Baseline contraction of myocytes from endotoxin-treated animals was reduced to 3.0 ± 0.3% (n = 17, $P <$ 0.001). The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) had no effect on myocytes from control animals, but it increased the contraction of myocytes from endotoxin-treated animals by 40% (fractional shortening increased to 4.3 ± 0.4%, $P < 0.01$). Similar results were obtained with N^G-methyl-L-arginine. The effect of L-NAME could be reversed by excess L-arginine, but not D-arginine. The effect of endotoxin was abolished by dexamethasone pretreatment. Methylene blue also reversed the effects of endotoxin but had toxic effects on myocytes. Agents that either prevent synthesis or the effects of NO reverse the depression of myocyte contraction seen following endotoxin treatment.

lipopolysaccharide; nitric oxide synthase; N^G-nitro-L-arginine methyl ester; endotoxic shock

ENDOTOXIC SHOCK is characterized by profound sepsis, hypotension, multiple organ failure, and extremely high mortality. In the early stages cardiac output is increased as peripheral resistance decreases, but later myocardial contractility itself declines, for reasons that are not well understood (4, 11, 13, 16). Recent evidence suggests that the fall in peripheral resistance, which is refractory to vasoconstrictors, is caused by the induction of nitric oxide (NO) synthase (9). Two forms of NO synthase have been identified (9). The first is the constitutive, calcium-dependent enzyme, which synthesizes the major component of endothelium-derived relaxing factor. The second, which is induced by endotoxin or cytokines, predominates in vascular smooth muscle with much lower levels in endothelium. This second form can be inhibited by corticosteroids. In both patients with septic shock (12) and in animal models (10, 15, 17, 19), it has recently been demonstrated that inhibition of this enzyme with the NO synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) causes dose-dependent increases in blood pressure and systemic vascular resistance. Thus induction of NO synthase may represent a common pathway by which different inflammatory mediators in endotoxic shock cause vasodilatation (10).

With the use of a citrulline assay, the inducible form of NO synthase has been detected in cardiac ventricular myocytes isolated from endotoxin-treated rats. Endotoxin also caused a corresponding elevation in guanosine 3′,5′-cyclic monophosphate (cGMP) content of the myocardium (14). Preliminary evidence from our laboratory has shown that the contraction of normal cardiac myocytes can be attenuated by nitric oxide derived from either endothelium or from sodium nitroprusside (2, 3). The hypothesis tested in the present study is that production of NO within the myocytes themselves contributes to the reduced cardiac contractility seen in endotoxic shock. This study was designed to examine whether contractility of cardiac myocytes was reduced by endotoxin treatment of animals, and if so, whether inhibition of the synthesis or transduction of NO would modify this attenuation.

METHODS

Guinea pig cardiac ventricular myocytes were isolated by enzymatic digestion, as described previously (6). A drop of myocyte suspension was placed in a 200-μl perspex chamber on the stage of an inverted microscope. The suspension was superfused at 2 ml/min with 2 mM calcium Krebs-Henseleit (KH) buffer (containing in mM: 119.1 NaCl, 4.7 KCl, 0.94 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.5 glucose) and bubbled with 95% O_2-5% CO_2 at 32 ± 0.5°C. Temperature was monitored by a thermistor in the cell bath. Cells were electrically stimulated to contract using a bipolar stimulator delivering 30 V/4 ms pulses at 0.5 Hz.

Contractility of individual myocytes was determined as described in detail elsewhere (7). Briefly, contraction amplitude and velocity of shortening of electrically stimulated myocytes were recorded using a video microscopy-length detection system and analyzed by computer-signal averaging. This system had a time resolution of 20 ms and a spatial resolution of 1 in 256, which allowed typically 10–15 sampling points within a single contraction and relaxation. Six consecutive contractions were signal averaged to produce data under each particular set of conditions. Myocyte contraction amplitude was calculated as a percentage of the resting length, and time to peak contraction and time from peak contraction to return to 90% of resting length (90% relaxation time) were recorded. Criteria were defined as previously described (6) to identify healthy, viable,

isolated myocytes: *1*) only rod-shaped cells without sarcolemmal blebs were examined; *2*) cells that exhibited spontaneous contractions were excluded; *3*) cells that displayed a variable baseline contraction to electrical stimulation at 2 mM calcium were rejected.

Animals treated with endotoxin (lipopolysaccharide, *Escherichia coli* serotype 055:B5; Sigma, Poole, Dorset, UK) were administered (4 mg/kg) intraperitoneally, 4 h before the animals were killed. In some cases, an intravenous injection of dexamethasone (4 mg/kg; David Bull, Warwick, UK) was given 1 h before injection of endotoxin.

Once stable contraction had been achieved, myocytes were exposed for 10 min to NO synthase inhibitors and substrates, and values obtained were compared with the mean of control contractions measured both before and after exposure to each drug. All recordings were made within 45 min of stabilization to avoid the decline in myocyte contraction amplitude, which is seen in experiments lasting longer than 2–3 h. Some experiments were performed in the presence of the guanylate cyclase inhibitor, methylene blue (David Bull Laboratories). In these experiments, recordings were made after 15 min of exposure and compared with baseline before administration. The NO synthase inhibitors L-NAME and L-NMMA and substrates L-arginine and D-arginine were obtained from Sigma.

Statistical differences between contraction of control myocytes and myocytes from endotoxin-treated animals, and differences between baseline contraction and contraction under different pharmacological conditions, were tested using Student's two-tailed t test for paired data. Values are expressed as means ± SE.

RESULTS

Effect of endotoxin treatment on contraction amplitude of cardiac myocytes. Figure 1 shows the effect of endotoxin treatment on myocyte shortening. The contraction amplitude of myocytes isolated from endotoxin-treated animals was reduced by 46% ($P < 0.001$) compared with cells from control animals. The reduced contractility of myocytes from endotoxin-treated animals was partly reversed by the nitric oxide synthase inhibitor L-NAME, whereas L-NAME had no effect on control cells. Pretreatment with dexamethasone abolished the effects of endotoxin. Addition of L-NAME to cells from animals treated with both dexamethasone and endotoxin had no further effect (fractional shortening 5.0 ± 0.9%; $n = 7$, P = NS, compared with normal cells).

Exposure of cells from endotoxin-treated animals to 5×10^{-6} M methylene blue for 15 min increased contraction amplitude substantially by 157 ± 27% (fractional shortening increased from 2.3 ± 0.4% of cell length to 6.1 ± 1.4% of cell length; $n = 7$, $P < 0.005$). Interestingly, exposure of normal control myocytes to methylene blue also increased contraction amplitude at 15 min (by 54 ± 11%, $n = 8$, $P < 0.01$). In experiments with myocytes from both normal and endotoxin-treated animals, some cells showed signs characteristic of calcium overload with hypercontractility and fibrillation, after a 20-min superfusion with methylene blue. Four of seven myocytes from endotoxin-treated animals and four of eight normal myocytes failed to return to baseline contraction after exposure to methylene blue.

Endotoxin treatment had no effect on either time to peak contraction or 90% relaxation time compared with normal controls. Time to peak contraction was 0.10 ± 0.02 vs. 0.12 ± 0.02 s, relaxation time 0.32 ± 0.02 vs. 0.30 ± 0.03 s, and values are means ± SE of 9 normal controls and 17 endotoxin-treated animals. Addition of L-NAME had no effect on either of these parameters (data not shown). Dexamethasone itself had no effect on myocyte contraction in control studies of normal cells (data not shown).

Effect of substrates and inhibitors of NO on myocyte contraction amplitude. Figure 2 shows the effects of different substrates and inhibitors on contraction of myocytes from endotoxin-treated guinea pigs. Addition of 10^{-4} M N^G-nitro-L-arginine methyl ester (L-NAME) to myocytes from endotoxin-treated animals increased contraction amplitude by 40 ± 6.6% ($P < 0.001$, $n = 17$).

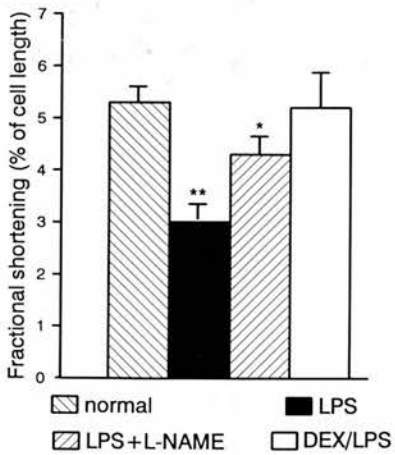

Fig. 1. Effect of endotoxin treatment on fractional shortening of cardiac myocytes. LPS, lipopolysaccharide (4 mg/kg ip) injection 4 h before death; L-NAME, N^G-nitro-L-arginine methyl ester 10^{-4} M; Dex, dexamethasone (4 mg/kg iv) injection 5 h before death. Values are means ± SE of 32 (normal), 17 (endotoxin-treated animals), and 7 (endotoxin + dexamethasone) myocytes. ** $P < 0.001$; * $P < 0.05$, compared with normal myocytes.

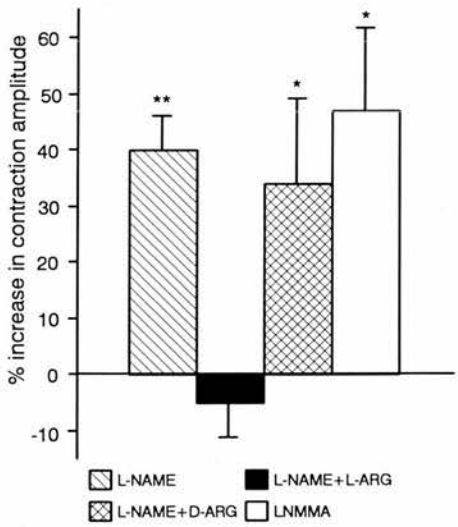

Fig. 2. Percentage change in contraction amplitude induced by substrates and inhibitors of nitric oxide (NO) synthase on cardiac myocytes from endotoxin-treated guinea pigs. L-Arg, L-arginine 10^{-3} M; D-Arg, D-arginine 10^{-3} M; L-NMMA, N^G-monomethyl-L-arginine 10^{-4} M. Values are means ± SE of 17 (L-NAME), 8 (L-arginine + L-NAME), 6 (D-arginine + L-NAME), and 5 (L-NMMA) experiments. ** $P < 0.01$; * $P < 0.05$, compared with baseline measurements.

Contractility returned to baseline within a 10-min washout period. N^G-methyl-L-arginine gave similar results. The effect of L-NAME was reversed by coadministration of L-arginine (10^{-3} M) but not D-arginine. L-NAME (10^{-4} M) had no effect on contraction of normal myocytes isolated from control animals (fractional shortening differed by $0.2 \pm 0.3\%$; $n = 7$, P = NS).

DISCUSSION

We have shown that endotoxin treatment of guinea pigs causes a substantial reduction in the contractility of isolated cardiac myocytes. About half of this reduction can be reversed by specific NO synthase inhibitors, and their effect can be overcome by an excess of L-arginine, the L-isomer amino acid from which NO is generated, but not by the inactive D-isomer. These results suggest that NO is being produced within the myocytes themselves and that this NO has an attenuating effect on myocyte contraction.

Pretreatment with dexamethasone prevents the induction of inducible NO synthase within cardiac myocytes (14). In the present study prior administration of dexamethasone prevented the reduction in contractility caused by endotoxin, and subsequent addition of L-NAME had no effect. This is further evidence for inducible NO synthase within the heart contributing substantially to the reduction in contractility occurring in endotoxic shock. Normal cardiac myocytes are reported to contain detectable amounts of constitutive NO synthase (14), although this does not appear to generate effective levels of NO, since in the present study and in a previous one from our laboratory (1), NO synthase inhibitors had no effect on contractility of cardiac myocytes isolated from healthy animals.

NO acts within cells by stimulating soluble guanylate cyclase to increase levels of cGMP, and this can be inhibited by methylene blue. We have shown previously that the contraction-attenuating effect of the NO donor sodium nitroprusside on normal myocytes can be reversed by methylene blue (3). Exposure to methylene blue in the present study caused a large increase in contraction amplitude of cells from endotoxin-treated animals. Interestingly, contraction of normal myocytes was also increased, although to a lesser extent. In both groups of cells administration of methylene blue caused some of the myocytes to become hypercontractile, with contractions characteristic of calcium overload. It may be that exposure to methylene blue causes progressive injury to cardiac myocytes, perhaps by increasing the permeability of the cells to extracellular calcium. We recorded contraction amplitude after a 15 min exposure to methylene blue, because an appreciable inotropic effect was usually observed by this time. The explanation why contractility of some of the cells did not return to baseline when methylene blue superfusion was discontinued is not clear. Whether the effect of methylene blue is limited to changes in cGMP concentration, or whether methylene blue has a direct toxic effect, is not explained by our study.

NO synthase inhibitors did not completely reverse the effect of endotoxin. This suggests that factors other than NO might contribute, although we cannot be sure that NO production was blocked completely by the NO synthase inhibitors. While endotoxin may itself be cytotoxic to myocytes, many other inflammatory mediators are released in endotoxic shock. Recent attention has focused on the use of anti-tumor necrosis factor (TNF) antibodies in septic shock, which appears to improve left ventricular function in such patients (18). In our study the effect of lipopolysaccharide was abolished by pretreatment with dexamethasone. Steroids not only prevent the induction of NO synthase by endotoxin but also prevent the generation of many inflammatory cytokines, which might impair myocyte contractility. Such cytokines may themselves act by induction of NO synthesis within the heart; in a recent study the cytokines TNFα, interleukin-2, and interleukin-6 inhibited myocardial papillary muscle contraction by a NO-dependent mechanism (5).

In animal models of endotoxic shock, recent studies have shown that NO synthase inhibitors were ineffective in small doses, whereas larger doses caused intense peripheral vasoconstriction and cardiovascular collapse (10, 19). It appears that at high doses inhibition of both the normal, constitutive NO synthase and the inducible NO synthase occurs, and complete inhibition of endogenous NO synthesis then allows unopposed peripheral vasoconstriction, deleterious in endotoxic shock (10, 19). The authors suggested that either coadministration of a NO donor with a NO synthase inhibitor or selective inhibition of the inducible form of NO synthase might be of benefit in this condition.

Our study is the first to show direct loss of contractility of cardiac myocytes in endotoxic shock, associated with the induction of NO synthase and production of NO within the myocytes. Contractility was restored partially by NO synthase inhibitors in keeping with two recent clinical case reports, where administration of NO synthase inhibitors was associated with a short-term increase in both blood pressure and cardiac ouput (12). Methylene blue was shown in another case report to be of short-term benefit in liver failure, and this benefit was considered to be due to inhibition of NO in the circulation and also possibly within the heart (8). In the present study we have found methylene blue to be toxic to myocytes after prolonged administration, although the reasons for this effect are not yet clear. The loss of contractility after endotoxin exposure can be prevented by pretreatment of animals with dexamethasone, but it is generally accepted that steroid therapy is of no benefit to patients with endotoxic shock. Until a specific inhibitor of inducible NO synthase is available, careful titration of a NO synthase inhibitor, perhaps combined with a NO donor, may be a possible treatment in endotoxic shock, not only by reducing the profound peripheral vasodilatation (19) but also by augmenting myocardial contraction.

In summary, we have shown that contractility of cardiac myocytes from endotoxin-treated animals is substantially reduced. This appears to be due to induction of NO synthase and subsequent production of NO within the myocytes themselves, since inhibitors of NO and NO synthase can reverse this reduction. Endotoxic shock is associated with myocardial depression, exacerbating the

profound hemodynamic changes that occur in this condition. NO production within cardiac myocytes may contribute to the contractile impairment in endotoxic heart failure.

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REVIEW

Circulatory failure in septic shock

Nitric oxide: too much of a good thing?

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Septicaemia and accompanying septic shock account for a substantial number of hospital deaths, despite appropriate antibiotic and supportive therapy. In the United States an estimated 100 000 patients die from sepsis in hospital each year.[1] One of the characteristic features of septic shock is profound hypotension caused by a decrease in peripheral vascular resistance. This hypotension is unusually resistant to both volume replacement and vasoconstrictor agents. In the earliest stages of spetic shock stroke volume and cardiac output are maintained or even increased; later, ventricular dilatation develops with a reduction in ejection fraction.[2-4] If patients survive, ventricular size and function return to normal as the infection is controlled and circulatory function restored. Recently, important advances have been made in our understanding of the pathophysiology and treatment of this frequently lethal condition.

Gram negative bacteria account for approximately 30% of cases of septic shock.[5] Bloodborne infection liberates endotoxin, the lipopolysaccharide component of the bacterial cell wall, into the circulation. Endotoxin and the organisms themselves activate host defense and inflammatory systems, including the complement, kinin, and coagulation cascades; the interleukins; tumour necrosis factor (TNF) and other endogenous mediators of inflammation; leucocytes; and platelets. These together generate the acute inflammatory response to bacteraemia. Although many inflammatory mediators are themselves vasoactive, recent work has shown that the hypotension and cardiac depression of septic shock are mediated by important changes within the vascular and cardiac muscle cells themselves.

Nitric oxide biology

Endothelium-derived factors, predominantly nitric oxide, modulate blood flow within the vasculature (for review see Moncada *et al*).[6] Nitric oxide, synthesised from the amino acid L-arginine by a constitutive nitric oxide synthase enzyme, is present in endothelial cells. It is released tonically in small amounts to act on adjacent vascular smooth muscle, causing relaxation and vasodilatation. This mechanism exists in health to regulate blood flow within tissue.[6 7] Endothelium-derived nitric oxide also inhibits platelet adhesion and aggregation, maintaining an antithrombotic luminal surface.

Nitric oxide synthase is also constitutively expressed in other cell types. These include some neural tissues; circulating neutrophils, mast cells and platelets; pancreatic islet β cells; and renal macula densa cells.[6 8] Production of nitric oxide seems to be a fundamental and important mechanism of intercellular signalling in these cell types. By its nature, nitric oxide is an unstable free radical with powerful oxidant properties when high local concentrations are achieved. Macrophages produce large amounts of nitric oxide when activated and this accounts for much of their cytotoxicity against micro-organisms and tumour cells.[8-10] Nitric oxide also damages iron-containing enzymes, for example NADH, and can inhibit DNA synthesis in some tumour cells.[8]

Abnormalities of nitric oxide production in endotoxic shock

In endotoxic shock the presence of disseminated foreign antigen, together with the inflammatory response, causes an inducible nitric oxide synthase to be generated in many other cell types which do not normally express this enzyme, including hepatocytes, fibroblasts, and vascular smooth muscle.[6 8] Subsequent production of large quantities of nitric oxide leads not only to haemodynamic instability but also to widespread production of nitric oxide-based free radicals which have the potential to cause considerable damage to tissues. Evidence from clinical studies supports this. Patients with endotoxic shock[11] and cancer patients receiving interleukin-2 (IL-2) chemotherapy, a cytokine which activates other endogenous cytokines to induce nitric oxide synthase,[12] excrete high concentrations of nitric oxide metabolites.

Vascular smooth muscle is itself not a source of nitric oxide in health. In endotoxic shock production of nitric oxide occurs within the muscle layer of the vessel wall and this causes excessive vasodilatation and hence a reduction in peripheral vascular resistance.[6] Analogues of the substrate L-arginine have been developed that act as substrate inhibitors of nitric oxide synthase. These cause systemic vasoconstriction and a pressor response in healthy animals by inhibiting constitutive nitric oxide production by the

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endothelium. In animals with experimental endotoxic shock inhibitors of nitric oxide synthase reverse hypotension but also cause a sustained increase in systemic vascular resistance, and at higher doses a decrease in cardiac output.[13–15] A reduction in cardiac output has also been seen with these agents in healthy animals.[13 16 17] Whether this fall in cardiac output is secondary to the rise in vascular resistance or to a direct effect of nitric oxide synthase inhibitors on cardiac contractility was not established by these studies.

Cardiac failure in endotoxic shock

Global deterioration of myocardial contractile function in patients with endotoxic shock has been established by clinical and radionuclide studies.[2–4] In healthy volunteers administration of purified endotoxin causes reversible depression of left ventricular function, in addition to the expected reduction in systemic vascular resistance.[18] Until recently, the cause of myocardial depression in endotoxaemia was considered to be a direct effect of endotoxin or an inflammatory mediator on myocardial tissue. The existence of a specific circulating myocardial depressant substance in endotoxic shock has been postulated but not proven.[4] Whereas coronary perfusion abnormalities in patients with coexisting cardiac or coronary disease and endotoxic shock may account for segmental abnormalities of left ventricular function, in patients with global myocardial impairment and endotoxic shock the loss of function cannot be wholly explained by changes in coronary flow.[4] As in the peripheral vasculature, multiple factors exist which depress cardiac function in endotoxic shock. However, there may be a common pathway for such mediators to impair myocardial contraction.

Overproduction of nitric oxide in the peripheral vasculature accounts for the vasodilatation and loss of vascular control in endotoxic shock. The hypothesis that overproduction of nitric oxide within cardiac muscle contributes to impaired function has now been tested. Studies of papillary muscles and individual cardiac myocytes isolated from healthy animals have shown that nitric-oxide-donating drugs[19 20] or nitric oxide itself released from adjacent endocardium or endothelium[19 21 22] can modulate the contractility of adjacent cardiac myocytes. In health cardiac myocytes do not produce appreciable amounts of nitric oxide.[23 24] In experimental endotoxaemia, or after administration of inflammatory cytokines to isolated myocytes, nitric oxide synthase enzyme is induced within cardiac myocytes.[25] This activity and the subsequent generation of nitric oxide within the myocytes themselves[25] is accompanied by a substantial loss of contractile function, compared with myocytes from healthy animals.[24] Both the generation of nitric oxide by myocytes[25] and their depression of contractility[24] can be reversed by specific inhibitors of the nitric oxide synthase enzyme. Pretreatment of animals with high dose corticosteroids prevents the induction of this enzyme and blocks completely the impairment of contraction.[24 25] The cytokine TNFα and the interleukins IL-6 and IL-2 can cause marked depression of cardiac contraction in isolated papillary muscles.[26] This effect is mediated by nitric oxide generation within the cardiac muscle itself.[26] Furthermore, there is overproduction of nitric oxide within the coronary microcirculation in experimental endotoxic shock.[27] This may not only perturb coronary blood flow in the microvasculature but may also depress cardiac contraction, since current work has shown a moderating influence of nitric oxide derived from vascular endothelium on adjacent cardiac myocyte contraction.[22]

Thus there seems to be a common mechanism in endotoxic shock contributing to both cardiac and vascular dysfunction. As in the peripheral vasculature, overproduction of nitric oxide within the myocardium, at least in experimental models, contributes importantly to the loss of contractility seen in patients with endotoxic shock.

Therapeutic strategies for the future

The cornerstones of treatment in septic shock are appropriately selected antibiotics and intensive support therapy. Molecular approaches to treatment accompanied by greater understanding of the pathophysiological mechanisms involved in endotoxic shock have generated important additions to existing therapy. Experimentally, pretreatment of animals with corticosteroids prevents the induction of the nitric oxide synthase enzyme within the heart and the vasculature.[24 25 28] In the clinical setting patients present with established infection, too late for steroids to be of benefit. This may explain in part the results of previous clinical trials which showed no benefit from corticosteroids in this condition.[5 29 30]

New developments in the treatment of endotoxic shock include a specific human monoclonal IgM antibody to the lipid A domain of endotoxin,[31] although distribution of this agent has recently been suspended worldwide pending further clinical trials. Another new therapy is TNF antibody. In a pilot study inhibition of this mediator of inflammation was shown to improve left ventricular function in patients with septic shock.[32] Whether there is also improvement in mortality has not been demonstrated.

Excess nitric oxide in the cardiovascular system seems to have a central role in endotoxic shock, but existing inhibitors of the nitric oxide synthase enzyme are not specific for the inducible form of the enzyme which accounts for this overproduction. In animal models of endotoxic shock a small dose of inhibitor blocked the excess production of nitric oxide but higher doses inhibited normal, endogenous nitric oxide production as well,[13–15] causing intense vasoconstriction and cardiovascular collapse. The vasoconstriction can be modulated by coadministration of nitric-oxide-donating agents (drugs which act like sodium nitroprusside) to replace the

nitric oxide normally present in the vasculature. An inhibitor specific for the inducible nitric oxide synthase enzyme would be a potential treatment but is not available. Widespread inhibition of nitric oxide has potentially serious side effects, for example impairment of the normal anticoagulant status of endothelium, and alteration of neurotransmission. Moreover, a degree of vasodilatation may in fact be valuable, allowing washout of toxic oxides of nitrogen from tissues.[6]

Encouraging results have been described in two recent clinical reports in which the non-specific inhibitors were used.[33 34] In two patients with septic shock, administration of L-arginine analogues (drugs which compete with L-arginine for the nitric oxide synthase enzyme) caused short-term increases in blood pressure. Cardiac output was increased in one patient and decreased in the other.[33] A further intriguing report described short-lived improvement in a patient with hepatic failure after administration of methylene blue, an agent that inhibits the effects of nitric oxide on vascular tone.[34] Methylene blue does not inhibit the cytotoxic effects of nitric oxide. Placebo controlled studies of nitric oxide synthase inhibitors in patients with septic shock are underway.

Conclusion

One of the principal mechanisms causing cardiovascular depression in endotoxic shock is an excessive production of nitric oxide both in the heart and in the vasculature. Research into the basic mechanisms of disease, in this case endotoxic shock, has led the way to potentially major advances in clinical practice. Results from current trials are awaited.

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Editorial Review

Inflammatory injury in myocardial ischaemia

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INTRODUCTION

The presence of inflammatory cells in infarcted myocardium was first described more than 50 years ago [1]. The importance of inflammatory processes in successful healing after myocardial infarction was revealed more recently when methylprednisolone given to post-infarction patients caused a substantial increase in both infarct size and deaths from ventricular rupture due to poor scar formation [2]. This indicated that inflammatory mechanisms are necessary for optimal repair after infarction. However, the possibility exists that such inflammatory processes may also damage nearby healthy or reversibly injured myocardium. Since thrombolysis and angioplasty have become so successful in relieving acute myocardial ischaemia, the role of acute inflammation in these early stages of myocardial injury has become the subject of intensive study.

There are experimental data to support the hypothesis that restoration of arterial flow to ischaemic myocardium may in fact contribute to, or hasten, cell death of some of the injured myocardial cells. However, not all authors agree that injury caused by ischaemia–reperfusion can be divided reliably into that caused by the initial ischaemic insult, and that resulting from subsequent inflammatory processes. The possibility that such inflammatory responses may bring about repair 'at a cost', i.e. that viable myocardial tissue is consumed by the early repair process, will be discussed in this review. Current understanding of the underlying mechanisms will be described, in particular the contribution to myocardial injury of neutrophils, oxygen free radicals and complement factors. The concept of injured myocytes signalling their own destruction will be discussed, and an outline of possible therapy to augment the benefits of thrombolysis or acute intervention will be considered.

NATURAL HISTORY OF INJURY INDUCED BY ISCHAEMIA AND ISCHAEMIA–REPERFUSION

After irreversible coronary occlusion the earliest manifestations of necrosis can be demonstrated in myocyte organelles and contractile elements by electron microscopy within 1 h [3], although gross and microscopic changes do not become apparent until 6–8 h after the onset of ischaemia [4]. The features of coagulative necrosis then appear: after about 8 h infiltration by neutrophils occurs, with the development of a zone of granulation tissue at the margin of the infarct [1, 5]. The infarct heals by digestion of necrotic tissue by macrophages with subsequent scar formation, and the infarct zone is replaced gradually by fibrous tissue.

If reperfusion occurs after irreversible myocyte injury, a characteristic pattern of contraction-band necrosis appears rapidly, with myocyte swelling and condensation of myofibrillar protein, disruption of the sarcolemma and deposition of calcium within the mitochondria [6–9]. Ionic changes with a massive influx of calcium into the cytoplasm occur and the myocytes are killed both by cellular disruption from hypercontraction, as well as loss of normal aerobic metabolism. Large numbers of neutrophils accumulate in the dead myocardium [10], although whether they are involved in killing myocytes alive at the time of reperfusion is uncertain.

After periods of up to 3 h of ischaemia, reperfused myocardium is characterized by the early accumulation of neutrophils, first within the blood vessels and then later within the injured myocardium [11, 12]. There is recovery of some of the injured myocytes and herein lies the central controversy of recovery after a short period of ischaemic injury: does the process of reperfusion with delivery of acute inflammatory components cause irreversible injury of myocytes which might have regained normal function, or does reperfusion merely hasten

Key words: adenosine, adhesion molecules, complement, endothelium, inflammation, leukotrienes, myocardial ischaemia, neutrophils, oxygen free radicals, reperfusion injury.
Abbreviations: ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin-8; LTB_4, leukotriene B_4; mRNA, messenger RNA; PAF, platelet-activating factor; sCR1, soluble complement receptor.
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the death of cells irretrievably injured? This question remains unresolved, but in the next section, evidence is presented that interference with some of these inflammatory processes reduces ultimate infarct size.

Short periods of myocardial ischaemia are accompanied by a reduction in contractile function, termed myocardial 'stunning' [13]. This appears to be due to alteration in the contractile function of myocytes, without evidence of microvascular obstruction by damaged endothelium or trapped neutrophils. There is recovery from myocardial stunning, and the time course of this recovery is related to the duration of ischaemia. After 1 min of coronary occlusion active shortening in the affected area returns within 20 s, and is complete by 30 min [13]. Regional loss of myocardial function after 15 min of ischaemia may take 60 min to recover. Even after prolonged ischaemia, some return of myocardial function in the affected area may be seen as late as 1–2 weeks after the event. For laboratory studies of reperfusion injury, periods of ischaemia of 30–90 min are usually employed so that a moderate degree of myocardial injury is achieved.

PATHOGENESIS OF INFLAMMATORY INJURY AFTER MYOCARDIAL ISCHAEMIA

There is abundant evidence implicating neutrophils as important mediators of injury in ischaemia–reperfusion damage. Depletion of circulating neutrophils by means of cytotoxic agents [14], anti-neutrophil antibodies [15] or leucocyte filters [16] are all reported to reduce myocardial infarct size in experimental models. The protective action of agents such as ibuprofen and prostacyclin in models of myocardial infarction [17, 18] is also associated with a reduction in neutrophil accumulation.

It should be borne in mind, however, that in a few studies some of these interventions have been reported to be without effect [11, 19]. This discrepancy in results may be due to methodological differences, for instance the manner in which tissue viability was assessed or whether myocardial blood flow was measured. Measurement of myocardial blood supply is of particular importance in canine models, which have been used for a large proportion of these studies. In this species, the myocardium has a variable, but often substantial, collateral blood supply. It is therefore of importance to measure the blood flow within the ischaemic region. In contrast, other animals, such as the rabbit, have virtually no collateral circulation, which is more akin to the situation in patients presenting with acute myocardial infarction in the absence of a long prior history of angina pectoris. Much of the controversy over interpretation of experimental findings from canine studies *in vivo* is due to dispute over the contribution made by collateral arteries.

Role of inflammatory mediators in neutrophil activation and accumulation

The role of inflammatory mediators in eliciting neutrophil infiltration into myocardial tissue after ischaemic injury has been the subject of considerable interest. Identification of the relevant mediators would provide an obvious target for modulation of neutrophil function. To date, a definitive role for any one mediator has not been assigned, although there is evidence implicating two well-established neutrophil chemoattractants, the complement fragment, C5a, and the eicosanoid, leukotriene B_4 (LTB_4).

There is a large body of experimental evidence indicating that activation of the complement pathway occurs after episodes of myocardial ischaemia, including alterations in the concentration of circulating complement components [20, 21]. This activation is central to the inflammatory response, since C5a which is generated during this process is one of the most potent neutrophil chemoattractants *in vivo*. The complement components C3, C4 and C5 have been detected immunohistochemically on the surface of myocytes in necrotic areas of baboon myocardium [22], whereas the terminal complement complex C5b-9 has been identified in the marginal zones of human infarcts [23]. Accumulation of radiolabelled C1q has also been reported in infarcted dog myocardium. Interestingly, co-localization of neutrophils in the same region of myocardium was observed in this study [24].

Evidence implicating complement activation in the inflammatory response has been provided by studies carried out using cobra venom factor, which depletes circulating complement. In experimental models of myocardial infarction, the use of cobra venom factor has resulted in a reduction in both myocardial infarct size and in the accumulation of neutrophils [22, 25]. More recently, a soluble form of complement receptor 1 (sCR1) has been developed, which inhibits complement activation by binding the C3 and C5 convertases, resulting in proteolytic inactivation of C3b and C4b. In a rat model of myocardial infarction, sCR1 also reduced infarct size and neutrophil infiltration [26]. Treatment with cobra venom factor is also reported to reduce the neutrophil chemoattractant activity of coronary sinus blood after myocardial ischaemia, suggesting that a product of complement activation is involved [27]. Furthermore, neutrophil chemoattractant activity in post-ischaemic cardiac lymph can be neutralized by antibodies to C5a [28].

The eicosanoid LTB_4 also acts as a neutrophil chemoattractant *in vivo* [29, 30]. LTB_4 is a product of neutrophil activation and has been detected in increased quantities in infarcted myocardium [31]. In this study peak concentrations of the eicosanoid preceded maximal accumulation of neutrophils, suggesting that there may be a causal relationship. Experimental studies in which drugs inhibiting the

enzyme 5-lipoxygenase and consequently the generation of LTB_4 were administered have provided support for this proposal. Reduction in infarct size, as well as neutrophil accumulation, was observed in a number of models of coronary artery occlusion and reperfusion [31, 32]. However, many of these agents possess other properties such as free-radical-scavenging activity. More recently, compounds with LTB_4 receptor antagonistic activity have become available. However, the results reported with these agents have so far been mixed: one LTB_4 antagonist was reported to be effective in a rat model of ischaemia and reperfusion [33], whereas another was ineffective in a canine model of myocardial infarction [34].

Although there is evidence that both C5a and LTB_4 are involved in causing neutrophil infiltration, this does not preclude a role for other inflammatory mediators. The cytokine interleukin-8 (IL-8) is a recently identified inflammatory mediator [35, 36], which can also elicit neutrophil infiltration *in vivo* [37]. We have found that a homologue of IL-8 is generated *in vivo*, subsequent to the appearance of C5a, in a model of acute inflammation in the rabbit [38]. As yet there is no evidence for a role for IL-8 in myocardial infarction; however, it remains a potential candidate. Platelet-activating factor (PAF) is another possible mediator of inflammation. In a model of intestinal ischaemia and reperfusion, administration of a PAF receptor antagonist is reported to reduce leucocyte adhesion and extravascular migration [39]. Although PAF antagonists are also reported to reduce myocardial infarct size [40, 41], in a rabbit model of myocardial ischaemia we found WEB 2086, a potent PAF antagonist, to be without effect on neutrophil accumulation [42].

Mechanisms of tissue damage by neutrophils

Neutrophils have been suspected as the agents of tissue injury in a number of pathological states, such as rheumatoid arthritis and acute respiratory distress syndrome, as well as in myocardial infarction. This suspicion hinges partly on their ability to release a number of potentially injurious agents when activated. These fall into two main groups: reactive oxidizing chemicals and proteolytic enzymes.

The first of these are products of the membrane-bound NADPH oxidase enzyme, which catalyses the production of superoxide anions, hydrogen peroxide and hydroxyl radicals. Although these radicals are themselves able to react with a number of biological substrates, it is thought that another product of oxidative metabolism is of greater importance *in vivo*. Hydrogen peroxide combines with the enzyme myeloperoxidase, one of the major constituents of neutrophil granules, to form an enzyme–substrate complex that oxidizes halides, and in particular Cl^-, to produce highly reactive toxic products such as hypochlorous acid (HOCl) [43]. HOCl is now thought to be the major product of oxidative metabolism by neutrophils [44]. These free radicals are able to cause direct cellular injury, for instance by oxidizing membrane phospholipids, thereby increasing the fluidity and permeability of the membrane and reducing its integrity. In addition, they also affect the activity of neutrophil proteolytic enzymes as described below.

Neutrophils possess a number of enzymes stored within granules. Three of these have been of particular interest with respect to tissue damage: the serine proteinase, elastase, and the two metalloproteases, collagenase and gelatinase. These enzymes are able to degrade key components of the extracellular matrix. Using endothelial monolayers *in vitro*, elastase has been shown to alter barrier properties and to cause detachment or even lysis of cells [45–47]. However, inhibitors of serine proteinases are present in the extracellular fluid in high concentrations in order to prevent inappropriate elastase activity. Furthermore, the metalloproteases are secreted in an inactive form [48]. Despite these safeguards, there is evidence to suggest that these enzymes are able to function at sites of inflammation [49]. Chlorinated oxidants are thought to inactivate α_1-proteinase inhibitor, thus destroying the anti-proteinase shield [50]. It has also been shown that activation of collagenase is dependent on the presence of a functional NADPH oxidase system [51]. It would appear therefore that by releasing free radicals in addition to proteolytic enzymes, the potential of neutrophils to cause tissue injury is considerably enhanced.

It has been reported recently that the collagen content of myocardial tissue decreases by 50% after 3 h of ischaemia in a rat model [52]. Whether this is due to neutrophil-derived proteases or to tissue procollagenase enzymes has yet to be determined. So far little work has been carried out into the effect of elastase or other proteases on myocardial injury. As selective and potent inhibitors become available this situation will no doubt change.

Tissue damage by oxygen free radicals

Since the 1970s it has been accepted that re-oxygenation of ischaemic myocardium may be associated with immediate and extensive ultrastructural damage, and the damage is caused, at least in part, by oxygen free radical species derived from the administered oxygenated medium [53]. Neutrophils also generate these reactive radicals, although to what extent oxygenated blood and neutrophils each contribute to the amount of free-radical damage has not been quantified. The role of oxygen free radicals in myocardial infarction has been the subject of a large number of studies using agents which can scavenge these radical species. As with studies into

the role of neutrophils the results of intervention to reduce free-radical damage have been mixed. Of 32 studies of interventions to reduce free-radical damage in animal models of myocardial injury, 16 showed benefit and 16 did not [54]. Although the scavenging agents catalase and superoxide dismutase can neutralize oxygen free radicals in the extracellular space, they are large molecules and do not readily enter cardiac myocytes [55]. Yet within myocytes, free radicals generated by the mitochondria cause damage to contractile proteins [56]. This may explain the modest protection afforded by scavenging agents in the published studies.

Effects of ischaemia on coronary microvasculature

One of the consequences of ischaemia is injury to the coronary microvasculature itself. Successful reperfusion of the large coronary arteries may not always be accompanied by restoration of blood flow within the area of injured myocardium. This has been termed the 'no-flow' phenomenon [57], and it appears to follow endothelial injury after periods of ischaemia greater than 60–90 min. Plugging of the microvessels by neutrophils during reperfusion contributes substantially to the no-reflow phenomenon, and is likely mediated both by neutrophil adhesion to damaged endothelium, together with neutrophils wedging in the microvessels. Such physical trapping occurs because activated neutrophils are less deformable, and hence their ability to squeeze through capillary lumina is impaired [58]. In the clinical situation, the no-reflow phenomenon occurs when restoration of patency is achieved by thrombolysis or angioplasty, yet reperfusion of myocardium in the ischaemic area remains impaired. Such impairment of myocardial perfusion is associated with a worse outcome, measured in terms of left ventricular ejection fraction, than patients in whom microvascular and hence myocardial tissue reperfusion is achieved [59].

Ultrastructural studies suggest that endothelium tolerates hypoxia better than cardiac myocytes, since zones of no-reflow are found only within areas of myocardial necrosis [60]. Endothelial cell swelling is seen as a result of ischaemic damage, but it only appears together with irreversible myocyte injury [57]. However, there is accumulating evidence that important alterations in endothelial function may occur much earlier in ischaemia, without evidence of morphological damage. Recent studies have shown that brief periods of ischaemia cause loss of endothelium-dependent vasodilatation and enhanced coronary vasoconstrictor responses [61], together with increased permeability of the coronary endothelium [62]. These functional changes occur without evidence of structural injury. Furthermore, administration of pharmacological agents which mimic endothelial products, including nitric oxide [63], iloprost (a stable analogue of prostacyclin) [64] and adenosine [65], has been shown to reduce experimental infarct size, probably both by replenishing protective endogenous autocoids lost during the ischaemic episode and also by inhibiting neutrophil–endothelial interactions. Such interventions may become clinically important, since limiting the no-reflow phenomenon may allow better myocardial perfusion after epicardial coronary artery occlusion–reperfusion.

MYOCARDIAL RESPONSE TO ACUTE ISCHAEMIA: CELLULAR EVENTS AND INTERCELLULAR SIGNALLING

Neutrophils have the potential to damage myocardium by their release of toxic products. Whether such toxins are liberated in a random fashion by activated neutrophils within an area of injured myocardium, or whether they are targeted towards injured myocytes in particular has not yet been determined. However, emerging evidence suggests that injured cardiac myocytes may signal their own destruction by activating such mechanisms at their cell surface.

Neutrophil–endothelial adhesion

For neutrophils to reach the interstitium and then the myocytes they have to adhere to and then traverse the endothelial barrier. At sites of inflammation neutrophils roll along endothelium by engaging and disengaging to the endothelial cells by adhesive interactions involving the neutrophil glycoprotein L-selectin (formerly termed LAM-1, Mel-14, LECAM-1, Leu-8) [66]. Tethering of neutrophils at sites of inflammation occurs via the expression of the endothelial glycoprotein P-selectin (formerly GMP-140, PADGEM, CD62), which appears on cell surfaces within minutes of endothelial activation by inflammatory mediators [67]. The rapid expression of P-selectin suggests that it may mediate early neutrophil adhesion to activated or injured endothelium, since it is expressed from intracellular or cell membrane stores without the need for synthesis of new proteins [67]. P-selectin interacts with its carbohydrate ligand CD15 on the neutrophil [68]. It appears that the neutrophils undergo an early change in the expression of their adhesion molecule presentation, with L-selectin exhibiting a rapid increase in affinity followed by down-regulation of its expression and the activation of the CD18 adhesion glycoprotein complex [69, 70].

Migration of neutrophils across the endothelial layer follows adhesion, and requires contact between the neutrophil integrin CD18 complex and the endothelial adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) [71]. Activated endothelium synthesizes and expresses the adhesion glycoproteins ICAM-1 and E-selectin (formerly endothelial–

leucocyte adhesion molecule-1). ICAM-1 is present constitutively on endothelium at low levels, but its expression is increased substantially by inflammatory mediators [72]. Monoclonal antibodies to the CD18 antigen have been shown to be very effective in reducing neutrophil infiltration into ischaemic-reperfused myocardium [42] and in reducing myocardial infarct size [73]. In the latter study a monoclonal antibody to ICAM-1 was also protective.

E-selectin only appears on the endothelium after stimulation [74] and requires protein synthesis *de novo*. The corresponding ligand for E-selectin on the neutrophil has been identified recently as another carbohydrate, sialylated Lewis X tetrasaccharide [75]. Both E-selectin and ICAM-1 mediate neutrophil adhesion to and migration through activated endothelium. It may be envisaged that after early initiation of neutrophil adhesion to ischaemic-reperfused endothelium by endothelial expression of P-selectin, further neutrophil–endothelial adhesion to damaged myocardium may be promoted by the ICAM-1/CD18 and E-selectin/sialylated Lewis X tetrasaccharide mediated interactions. This hypothesis is supported by current research. Very recently, increased production of messenger RNA (mRNA) for both P- and E-selectin has been demonstrated in the endothelium of blood vessels from the injured segments of ischaemic-reperfused hearts [76a, 76b].

Neutrophil–cardiac myocyte interactions

Once neutrophils have reached the extravascular space they can directly attack cardiac myocytes. Whether this damage is directed specifically towards previously injured myocytes is not known, but current research indicates that activated myocytes appear to exhibit signals which activate neutrophil wound repair mechanisms. Recent studies indicate that cardiac myocytes, as well as endothelial cells, express ICAM-1 in response to activation by inflammatory mediators [76c]. Neutrophils bind to ICAM-1 on activated myocytes by a process involving their adhesion molecule complex CD18 [77], and this receptor-mediated adhesion appears to be the main mechanism of neutrophil adherence to cardiac myocytes. Furthermore, mRNA for ICAM-1 can be induced in both endothelial cells and cardiac myocytes by post-ischaemic cardiac lymph [78], and recent data suggest that direct neutrophil-mediated injury of cardiac myocytes requires adhesion by the CD18/ICAM-1 mechanism [28]. However, expression of ICAM-1 by myocytes requires protein synthesis *de novo* [77]. Since it has been shown that complement activation products are present on the surface of myocytes after ischaemia, a possible mechanism of early neutrophil attachment is via a CD11b/CD18 (complement receptor 3) interaction with iC3b.

It is possible therefore that administration of monoclonal antibodies to CD18 or ICAM-1 is able to confer protection in models of myocardial ischaemia by inhibiting direct neutrophil–cardiac myocyte interactions as well as by inhibiting neutrophil migration through the endothelium. These recently published and ongoing studies provide exciting new evidence that injured myocardium plays an active role in the inflammatory response to ischaemic injury.

Myocardial defence against ischaemic injury

Poorly understood endogenous defence mechanisms exist within the heart which offer some myocardial protection against periods of ischaemia. Ischaemic preconditioning, where brief periods of ischaemia afford protection against a subsequent, more prolonged ischaemic episode, is a well-recognized phenomenon [79]. The tolerance to ischaemia is associated with reduced energy utilization during ischaemia [80], although whether this accounts for all the protection is not clear. The purine nucleoside adenosine, released by catabolism of ATP, appears to have a role in ischaemic preconditioning, since the beneficial effect of preconditioning can be abolished by adenosine antagonists [81].

Adenosine may also have other protective roles in ischaemic-reperfusion injury. Adenosine has widespread actions on endothelium, vascular smooth muscle, cardiac myocytes, neutrophils and platelets, in addition to its therapeutic pharmacological effects on conducting tissue. Adenosine possesses a number of actions which are likely to be protective during myocardial ischaemia. These include vasodilatation in response to hypoxia, which may improve local blood flow in ischaemic areas [82], as well as protecting against free-radical injury [82, 83]. Adenosine inhibits both the adhesion to endothelium and the release of superoxide anions by neutrophils [84, 85]. It has also been reported to inhibit platelet aggregation in ischaemic myocardium [86]. Some agents which reduce infarct size in experimental models are thought to act by augmentation of adenosine release [87] and administration of adenosine reduces myocardial reperfusion injury in dogs [88]. Endogenous adenosine is quickly washed out during reperfusion; therefore agents which mimic or augment adenosine release are of potential value in myocardial injury.

Heat shock proteins, induced by a prior exposure to hyperthermia and other stressful stimuli, are protective against subsequent ischaemic episodes [89]. However, the requirement for protein synthesis *de novo* [90] precludes a major role for these fascinating proteins in the immediate endogenous protection against myocardial ischaemia–reperfusion injury. It seems unlikely therefore that they are involved in ischaemic preconditioning.

CONCLUSIONS AND STRATEGIES FOR THE FUTURE

This review began with a description of the disasters caused by interference with healing processes after myocardial infarction [2]. Our understanding of the behaviour of inflammatory mechanisms has increased greatly since the study of Roberts *et al.* [2] was published. Early reperfusion is essential for effective tissue salvage after coronary occlusion. However, it is likely that reperfusion carries its dangers, in that blood defensive cells, notably the neutrophil in early stages, are programmed to target injured tissue cells. Therapeutic intervention is possible in order to abolish the accumulation of neutrophils. This may be beneficial in the short-term; however, the long-term effects (as indicated by the trial with anti-inflammatory steroids) are likely to be deleterious. This is because the acute inflammatory events trigger the process of tissue healing and remodelling, events that are necessary in order to conserve important physical properties of the ventricular wall. For this reason a detailed knowledge is required of the mechanisms involved in the inflammatory events occurring in ischaemia and reperfusion of the myocardium. Only with this knowledge will it be possible to design a rational intervention.

One possible strategy may be to attenuate neutrophil accumulation for a brief period at the start of reperfusion to disengage the phase of re-oxygenation from the initial phase of neutrophil accumulation. While some possible therapeutic agents, such as monoclonal antibodies to neutrophil adhesion molecules, are available, the size and cost of a carefully conducted, randomized trial would be substantial. Thrombolysis in myocardial infarction is successful, and the mortality in the large trials so low (10–11% [91]), that additional benefit over current strategies would require the study of many thousands of patients to test the ultimate efficacy and safety of such a therapy. When the cost of producing such agents in large quantities becomes economic, such studies may well be performed.

Encouragingly, however, new low-molecular-mass carbohydrate agents which bind to the oligosaccharide moieties of adhesion receptors, thus blocking their action, are cheaper to produce, easier to manufacture and potentially more readily administered to patients, and are nearing clinical trials. Another potential agent to reduce inflammation in myocardial injury is the sCR1 receptor, already shown to be of benefit in animal studies [26]. Prostacyclin analogues which switch off neutrophil function are another area of therapeutic interest, although hypotension is a problem [92]. In the near future, results of trials using drugs which act either as analogues of adenosine or stimulants of its release are expected, and these and other early modulations of the inflammatory response may offer important gains in the management of myocardial ischaemia.

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